BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF ESCITALOPRAM OXALATE IN HUMAN PLASMA BY USING RP-HPLC METHOD

Dissertation Submitted to

The Tamil Nadu Dr. M.G.R. Medical University, Chennai

In partial fulfillment for the requirement of the Degree of

MASTER OF PHARMACY

(Pharmaceutical Analysis)

OCTOBER-2016

DEPARTMENT OF PHARMACEUTICAL ANALYSIS

KMCH COLLEGE OF PHARMACY,

KOVAI ESTATE, KALAPATTI ROAD,

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This is to certify that the dissertation work entitled**" BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF ESCITALOPRAM OXALATE IN HUMAN PLASMA BY USING RP-HPLC "** is a bonafide work carried out by **Reg. No: 261430753**, student in Master of Pharmacy , Department of Pharmaceutical Analysis, KMCH College of Pharmacy, Coimbatore, Tamil Nadu, during the academic year 2015-2016.

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Date : **Signature of the Guide**

Place: Coimbatore

DECLARATION

I do here by declare that to the best of my knowledge and belief ,the dissertation work entitled **" BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF ESCITALOPRAM OXALATE IN HUMAN PLASMA BY USING RP-HPLC"** submitted to the Tamil Nadu Dr. M.G.R. Medical university , Chennai, in the partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmaceutical Analysis,** was carried out at Department of Pharmaceutical Analysis, KMCH College of Pharmacy, Coimbatore during the academic year 2015-2016 .

Date:

Place: Coimbatore **(Reg. No.261430753)**

EVALUATION CERTIFICATE

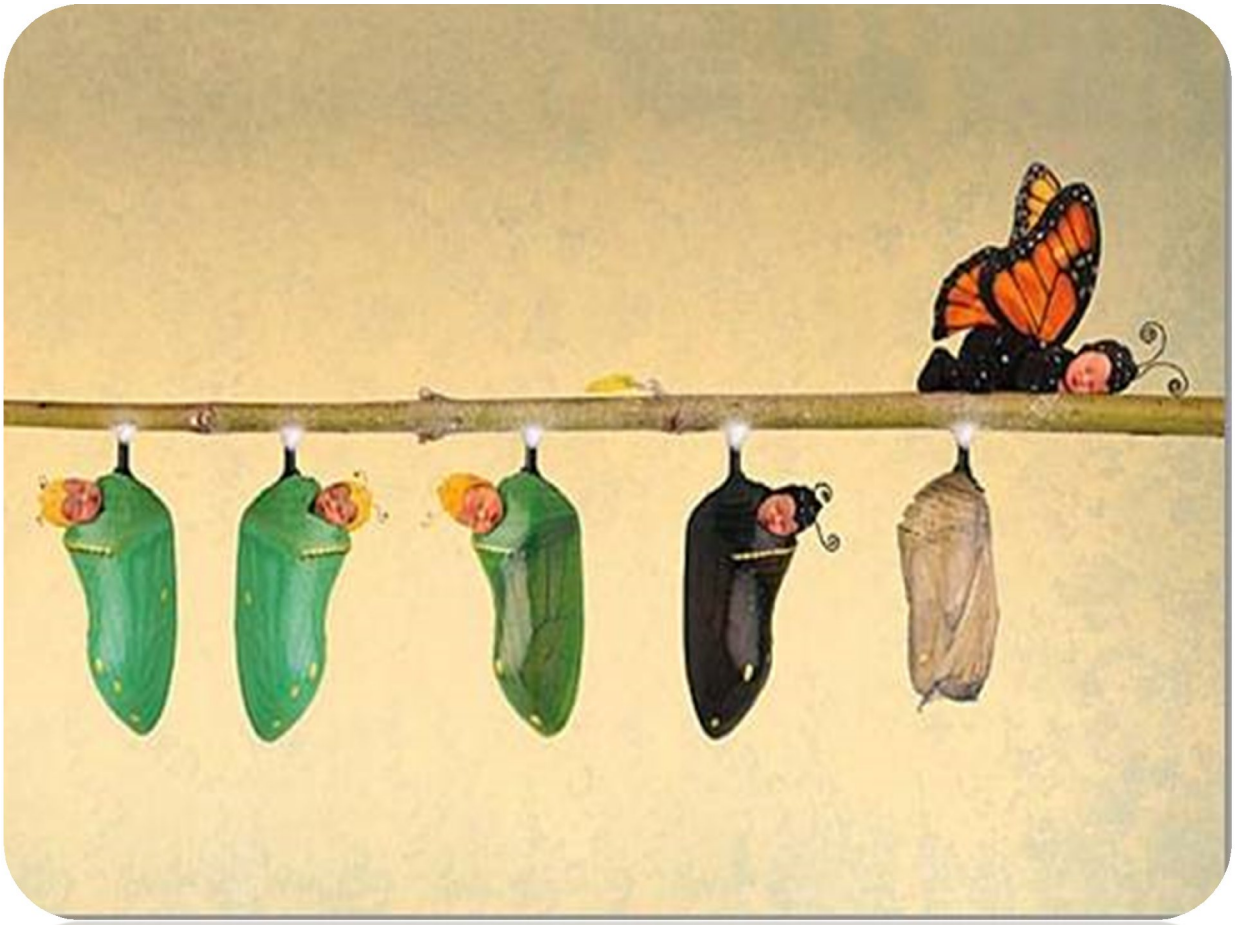
This is to certify that the work embodied in the dissertation work entitled **"BIOANALYTICAL METHOD DEVELOPMENT AND VALIDATION FOR THE ESTIMATION OF ESCITALOPRAM OXALATE IN HUMAN PLASMA BY USING RP-HPLC"** submitted by **Reg. No:261430753** to the Tamil Nadu Dr. M.G.R. Medical university , Chennai, in the partial fulfillment for the Degree of **Master of Pharmacy** in **Pharmaceutical Analysis,** is a bonafide research work carried out by the candidate at Department of Pharmaceutical Analysis, KMCH College of Pharmacy, Coimbatore during the academic year 2015-2016.

Examination Center: K.M.C.H College of Pharmacy, Coimbatore

Date:

Internal Examiner **External Examiner**

Convener of Examination



Dedicated to Almighty
My Parents, My Dear Sister
& Friends

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DEEPTHI VISAKH

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ABBREVATIONS

HPLC	High Performance Liquid Chromatography
UV	Ultra violet
BA	Bioavailability
MW.	Molecular weight
e.g.	Example
i.e.	That is
%	Percentage
PDA	Photo Diode Array
IS	Internal Standard
ACN	Acetonitrile
MET	Methanol
RF	Response Factor
mg	Milligram
mL	Milliliter
mM	Milli molar
µg	Microgram
w/w	Weight by weight
v/v	Volume by volume
µg/ml	Microgram per milliliter
ng /ml	Nanogram per milliliter
pH	Hydrogen ion concentration
^{0}C	Degree centigrade
T	Time

Abs.	Absorbance
Conc.	Concentration
Fig.	Figure
Tab.	Table
M.P.	Melting Point
AUC	Area under curve
CV	Coefficient of variance
RSD	Relative standard deviation
L/h	Liter per hour
L/kg	Liter per kilogram
Rpm	Rotation per minute
SP	Stationary Phase
MP	Mobile Phase
λ max	Wavelength maxima
Rt	Retention time
SPE	Solid Phase Extraction

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ABSTRACT

A simple, precise, accurate, rapid and sensitive reverse –phase High Performance Liquid Chromatography method for the estimation of Escitalopram oxalate in human plasma was developed and validated. Sample preparation was carried out using solid phase extraction (SPE) method. The separation of Escitalopram in biological fluid with the internal standard Tinidazole was achieved by Enable C_{18} column using 0.2% ortho phosphoric acid and acetonitrile in the ratio of 65:35%v/v as mobile phase at a flow rate of 1.0ml/min at ambient temperature. Detection was carried out at 240nm. The retention time of internal standard and Escitalopram was found to be 4.7 and 10.5 min respectively. The standard curve was linear (R^2 >0.995) over the concentration range of 100-800 ng/ml. All the analytical validation parameters were determined as per ICH guidelines. The bioanalytical method developed was selective, robust and reliable as accuracy, precision, recovery and other validation parameters were within the limits as specified by the guidelines. The peaks obtained for the drug of interest and internal standard were well resolved from each other without any plasma interferences and the peaks were symmetrical in nature with acceptable tailing factor. The method can be very useful for the therapeutic drug monitoring (TDM), in bioequivalence studies, for pharmacokinetics study and also in toxicology and biomedical investigations.

Key words: RP-HPLC, Escitalopram oxalate, Solid phase extraction, Human plasma

INTRODUCTION

BIOANALYSIS [1-3]

Bioanalysis is the method used to determine the concentration of drugs, their metabolites and / or endogenous substances in the biological matrices such as blood plasma, serum, cerebrospinal fluid, urine and saliva. Bioanalytical methods are widely used to quantitative drugs and their metabolites in the physiological matrices and the methods could be applied to studies in areas of human clinical pharmacology and non human pharmacology / toxicology. Bioanalytical method employed for the quantitative determination of drugs and their metabolites in biological fluids plays a significant role in the evaluation and interpretation of bioequivalence, pharmacokinetics and toxic kinetic studies. It helps in carrying studies like pharmacodynamics, toxicology, pharmacokinetics, bioequivalence, therapeutic drug monitoring (TDM) and clinical studies. Initial stages these studies are done only to find out over dosage conditions and in toxicological studies. When concentration of drug in biological matrix is known, then pharmacokinetic parameters are calculated from that. Bioanalytical studies are important in drug discovery and development. So these studies are performed carefully.

Therapeutic efficacy of the particular drug can be known by bioanalysis. In pharma field bioanalysis plays a significant role .Bioanalysis involves the following steps.

- Selection and collection of biological fluid.
- Preparation of sample –Analyte extraction from biological matrix.
- Analyte detection done by various methods.

ANALYSIS OF DRUG IN VARIOUS BIOLOGICAL MEDIA [4, 5]

The most common samples obtained for biopharmaceutical analysis are blood, urine, and feces, especially if the drug or metabolite is poorly absorbed or extensively excreted in the bile. Other media that can be utilized includes saliva, breath and tissue.

The choice of sampling media is determined largely by the nature of used in the study. For example, drug levels in a clinical pharmacokinetic study demand the use of blood, urine, and saliva. A bioavailability study may require drug level data in blood and / or urine whereas a drug identification or drug abuse problem may be solved with only one type of biological sample.

Detection of drug or its metabolite in biological media is usually complicated by the matrix. Because of this, various types of cleanup procedures involved i.e. solvent extraction and chromatography are employed to effectively separate drug components form endogenous biological materials. The sensitivity and selectivity of the assay method was limited by the efficiency of the clean up methodology.

If the blood is allowed to clot and is then centrifuged, about 30 to 50% of the original volume is collected as serum (upper level). Plasma generally is preferred because of its greater yield from blood. Blood, serum or plasma samples can be utilized for bioanalytical studies and may require protein denaturation steps before further processes.

If plasma or serum is used for the analytical procedure, the fresh whole blood should be centrifuged immediately at 5000 rpm for approximately 5 to 10 min, and the supernatant should be transferred by means of a suitable device, such as pasture pipette, to a clean container of appropriate size of storage.

Urine is the easiest one to obtain from the patient and also permits collection of large and frequently more concentrated samples. The lack of protein in a healthy individual's urine obviates the need for denaturation steps, because urine samples are readily obtained and often provide the greatest source of metabolites, they are frequently analyzed in drug metabolism studies.

PREPARATION AND PRESERVATION OF BIOLOGICAL SAMPLES

Sample preparation is important step for analysis of drugs and metabolites in bioanalytical study. Biological samples contain proteins, various endogenous and exogenous substances that may interfere with analyte. The objective of sample preparation is to free analyte of interest from all possible unwanted substances without significant loss of analyte. When drugs are susceptible to plasma esterase, the addition of esterase inhibitors, such as sodium fluoride was immediately added after collection helps to prevent decomposition.

When collecting and storing biological samples, there are chances for the analyte to get contaminated with storage vessels , For example, plastic containers contains high boiling liquid bis (2-ethylhexyl) phthalate; similarly, the plunger-plugs may contain tri-butoxyethyl phosphate,

which can be interfere with certain drug analysis. Hence care should be taken in selecting the material of containers for sample storage and preservation.

ESTIMATION OF DRUGS BY BIOLOGICAL FLUIDS [6, 7]

The choice of sampling media is determined largely by the nature of the drug study. For example, drug levels in a clinical pharmacokinetic study demand the use of blood, urine, and possibly saliva. A bioavailability study may require drug level data in blood or urine. Steps involved in the estimation of drugs in biological fluid are collection of the sample, sample treatment and separation of the compound of interest from the matrix and analysis.

EXTRACTION PROCEDURE FOR BIOLOGICAL SAMPLE [8-10]

Sample preparation is a technique used to clean up a sample before analysis and /or to concentrate a sample to improve its detection. When samples are biological fluids such as plasma, serum or urine, this technique is described as bioanalytical sample preparation.

Objectives of Bio-analytical sample preparation:-

1. Removal of unwanted matrix components (primarily protein) that would interfere with analyte determination.
2. Concentration of analyte to meet the detection limits of the analytical instrument.
3. Exchange of the solvent or solution in which the analyte resides so that it is compatible with mobile phase for injection into a chromatographic system.
4. Dilution to reduce solvent strength or avoid solvent incompatibility.
5. Stabilization of analyte to avoid hydrolytic or enzymatic degradation.

After selection of biological fluid the required analyte should be extracted from it. This step in bioanalytical method is more important because sample preparation can be done by different methods of extraction .The sample preparation is a time taking process and it should be done carefully because of its importance. If biological matrix is in liquid state like blood, plasma and urine then liquid-liquid extraction is used or it is solid then liquid-solid extraction can be done.

Different types of extracting methods are

- Dilution followed by injection
- Solid Phase extraction (off line/online)
- Protein precipitation
- Filtration
- Liquid-liquid extraction
- Protein removal by equilibrium dialysis or ultrafiltration
- Restricted access media
- Solid-supported liquid-liquid extraction
- Monolithic columns
- Immunoaffinity extraction

Out of all these methods the most prominent and widely used techniques for extraction are:

- Protein precipitation method.
- Liquid-liquid extraction method.(LLE)
- Solid-phase extraction method.(SPE)

Protein precipitation [11, 12]

Protein precipitation is based on the interaction between the precipitation reagent and protein groups. Soluble proteins generally have a hydrophobic core surrounded by a hydrophilic surface including ionic groups that are not involved in intra-molecular binding. Organic solvents interfere with the intra-molecular hydrophobic interactions of proteins.

The addition of a volume of solvent (frequently acetonitrile) to the serum causes the proteins of the serum to precipitate and leaves the analyte of interest in the solvent, which can either be injected directly or dried down and reconstituted in a smaller volume to concentration before injection. While this is the fastest and simplest method for sample preparation, it is the most likely to cause ion suppression issues, especially in ESI, where the co-elution of endogenous compounds such as lipids, phospholipids and fatty acids affect the ESI droplet desolvation process.

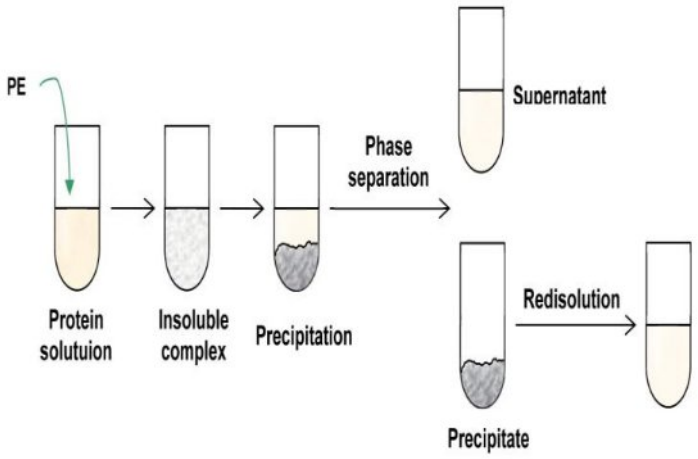


Fig 1: Schematic diagram of protein precipitation technique.

Procedure

a) **In case of acids:** Take 0.5 to 1.0 ml of plasma/serum/urine and add 100 to 200µl of 10 to 20 % perchloric acid or trichloroacetic acid or sometimes decreasing the volume and increasing the percentage of acid are also recommended.
b) **In case of organic solvents:** Take 0.5 to 1.0 ml of plasma and add 0.5 to 2.0ml of solvent methanol or acetonitrile.

After adding the acid or solvent vortex the vial for complete precipitation of protein then centrifuge and inject the supernatant. It is recommended to filter the sample whenever the technique is used to avoid clogging of the column.

Liquid-Liquid Extraction [13, 14]

Liquid- liquid extraction is a common technique used to extract analytes from liquid matrices Isolation of analyte from interferences is done by partitioning or distribution of the sample between two immiscible liquids or phases. Typically, with LLE one phase will be aqueous (often the denser or heavier phase) and the second phase is an organic solvent (usually the lighter phase). Hydrophilic compounds prefer the polar aqueous phase, whereas hydrophobic compounds will prefer the organic solvent. The two phases used in this method should be immiscible to each other. It is very useful for separating analytes from interferences by partitioning the sample between these two immiscible liquids or phases.

The basic principle is the tendency of an analyte to prefer one solvent over another immiscible solvent. The process is based on the Nernst distribution law, which states that any species will distribute between two immiscible solvents so that ratio of the concentrations remain constant. The often-quoted partitioning coefficient of a species is Log P which is defined as the ratio of the concentration in octanol divided by the concentration in water

Procedure

To the biological fluid two immiscible solvents are added and centrifuged, then the organic solvent is evaporated. To ensure rapid equilibrium more surface area is required that is achieved by thoroughly mixing or manually shaking. The residue thus obtained is reconstituted with the suitable solvent of small volume which is compatible with HPLC separation while analytes extracted in aqueous phase can be directly injected into HPLC. More than one sample can be extracted in this method. To do this method effectively some steps are present, they are,

- To facilitate removal of the extraction solvent at the end with low boiling point is used.
- Solvent with low viscosity is used to facilitate mixing with the sample.
- Analyte must be soluble in extracting solvent.
- The organic solvent selected should have low solubility in water, high purity and should be compatible for the HPLC detection techniques.

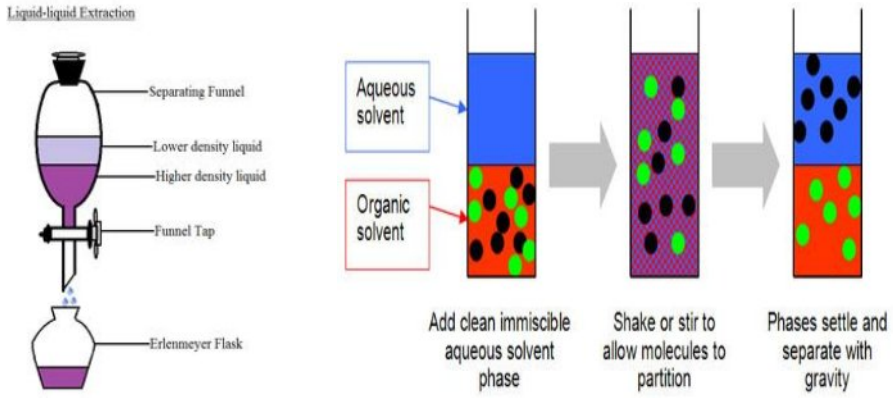


Fig 2: Schematic representation of LLE process

Solid Phase Extraction [15-19]

Solid-phase extraction (**SPE**) is a sample preparation process by which compounds that are dissolved or suspended in a liquid mixture are separated from other compounds in the mixture according to their physical and chemical properties. Solid phase extraction can be used to isolate analytes of interest from a wide variety of biological matrices. SPE uses the affinity of solutes dissolved or suspended in a liquid for a solid through which the sample is passed to separate a mixture into desired and undesired components. The result is that either the desired analytes of interest or undesired impurities in the sample are retained on the stationary phase. The portion that passes through the stationary phase is collected or discarded, depending on whether it contains the analytes or impurities. If the portion retained on the stationary phase includes the desired analytes, they can then be removed from the stationary phase for collection in an additional step, in which the stationary phase is rinsed with an appropriate eluent.

Solid–phase extraction method also contains two phases, one is solid and other is liquid phase. In this method analyte is retained on the solid phase, while sample passes through, followed by elution of an analyte with an suitable solvent. The solid phase used here is plastic disposable column or cartridge which is packed with sorbent like reversed phase material (C-18silica) which has been bonded a hydrocarbon phases. SPE involves a solid-liquid phase separation of the analytes from the biological sample, by the selective transfer between a liquid and solid state. The analyte is physically separated from the biological matrix by the differential interacting with a solid phase sorbent material. These sorbents, packed primarily into either disposable cartridges or discs, can be polar, non-polar or ionic depending on the experimental requirements.

The principle of SPE is similar to that of liquid-liquid extraction (LLE), involving a partitioning of solutes between two phases. However, instead of two immiscible liquid phases, as in LLE, SPE involves partitioning between a liquid (sample matrix or solvent with analytes) and a solid (sorbent) phase. This sample treatment technique enables the concentration and purification of analytes from solution by sorption on a solid sorbent and purification of extract after extraction. The general procedure is to load a solution onto the SPE solid phase, wash away undesired components, and then wash off the desired analytes with another solvent into a collection tube.

Mechanism of Solid Phase Extraction Process

Solid phase extraction involves four distinct steps, which is as follows:

a) Conditioning of the packing
b) Loading of the sample
c) Washing the packing/ sorbent
d) Recovery of the analyte

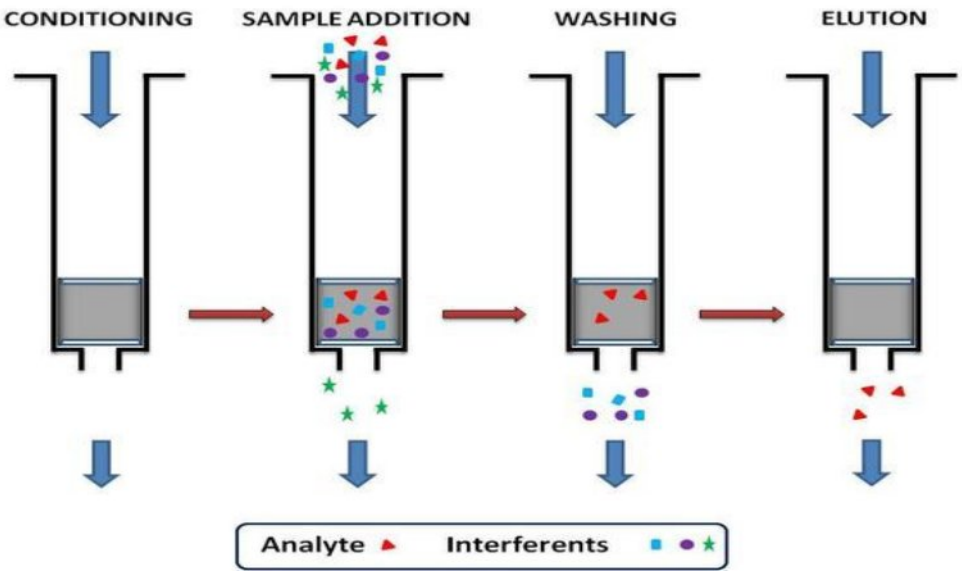


Fig 3: Solid phase extraction separation process

a) Conditioning of the packing

Prior to the addition of the sample conditioning and packing of the cartridge is done. The hydrophobic packing is dry initially and the ligands are all collapsed. Organic phase extends the ligands and prepares them for interaction with the analyte.

b) Loading of the sample

A solution containing the sample, as well as, any contaminants or interfering substances, is introduced onto the stationary phase by dissolving in a weak solvent. This weak solvent helps in retention of strong analyte. The stationary phase will interact with the analyte molecules, retaining them until the final elution step. As the separation of the analytes is achieved by the selective transfer between a liquid and solid state, the analyte is

physically separated from the biological matrix by the differential interacting with a solid phase sorbent material.

c) **Washing of the packing / sorbent**

Washing step aids in the removal of contaminants and interfering compounds. This step is conducted again with the solvent composition of the sample matrix. During this step, the analyte will be bound by the stationary phase and will not be released by the column.

d) **Recovery of the analyte**

An elution solvent is introduced to the column. The elution solvent will have distinctly different properties than the solvent used for the previous three steps. For example, considering a hydrophobic pharmaceutical compound should the sample be loaded in an aqueous plasma solution, an organic solvent such as acetonitrile may be used to elude the compound. Strong elution solvent is passed through the cartridge and it gives 100% collection of analyte from this method. The eluted solution collected may be evaporated in order to control the concentration.

SOLID PHASE EXTRACTION THEORY

How compounds are retained by the sorbent

1) Reversed Phase

   (Polar liquid phase, non polar modified solid phase)

   Hydrophobic interactions

   - Non polar-non polar interactions
   - Van der Waals or dispersion forces

2) Normal Phase

   (Non polar liquid phase, polar modified solid phase)

   Hydrophilic interactions

   - polar-polar interactions
   - hydrogen bonding
   - pi-pi interactions
   - dipole-dipole interactions
   - dipole-induced dipole interactions

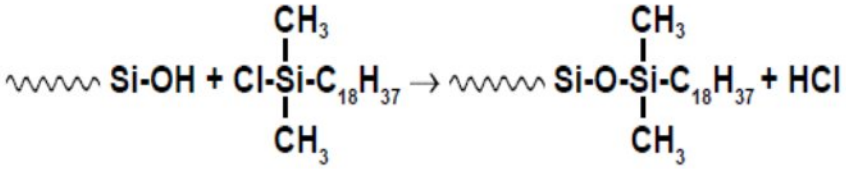
3) Ion Exchange

   Electrostatic attraction of charged group on compound to a charged group on the sorbent's surface

4) Adsorption (Interaction of compounds with unmodified materials)

Reversed Phase SPE

Reversed phase separations involve a polar or moderately polar sample matrix (mobile phase) and a non polar stationary phase. The analyte of interests will be typically non polar or less polar in nature. Several SPE materials, such as the alkyl- or aryl-bonded silicas (**LC-18, ENVI-18, LC-8, ENVI-8, LC-4,** and **LC-Ph**) are in the reversed phase category.



Here, the hydrophilic silanol groups at the surface of the raw silica packing (60Å pore size, 40µm particle size) have been chemically modified with hydrophobic alkyl or aryl functional groups by reaction with the corresponding silanes.

The Role of pH in solid phase extraction

Solutions used in SPE procedures have a very broad pH range. Silica-based packings, such as those used in HPLC columns, usually have a stable pH range of 2 to 7.5. At pH levels above and below this range, the bonded phase can be hydrolyzed and cleave off the silica surface, or the silica itself may dissolve. In SPE, the solutions are usually in contact with the sorbent for short periods of time and also since the SPE cartridges are disposable, and are meant to be used only once, any pH can be used to optimize retention or elution of analytes. If stability of the SPE cartridge at an extreme pH is crucial, then polymeric or carbon-based SPE materials may be used as these materials are stable over the pH range of 1-14.

For reversed phase SPE procedures on bonded silicas, if trapping the analyte in the tube is desired, the pH of the conditioning solution and sample should be adjusted for optimum analyte retention. If the compound of interest is acidic or basic we can use a pH at which the compound is not charged. Retention of neutral compounds is usually not affected by pH. Conversely, you

can use a pH at which the unwanted compounds in the sample are retained on the SPE packing, but the analyte of interest passes through unretained. For adsorption media that are used under reversed phase conditions, a pH should be chosen to maximize retention of analyte on the sorbent as with reversed phase bonded silica. Elution is usually done with an organic solvent, so pH is usually not a factor at this point.

Table 1 : Commonly used solvents in solid phase extraction

Polarity			Solvent	Miscible in Water?
Nonpolar	Strong Reversed Phase	Weak Normal Phase	Hexane	No
			Isooctane	No
			Carbon tetrachloride	No
			Chloroform	No
			Methylene chloride (dichloromethane)	No
			Tetrahydrofuran	Yes
			Diethyl ether	No
			Ethyl acetate	Poorly
			Acetone	Yes
			Acetonitrile	Yes
			Isopropanol	Yes
	Weak Reversed Phase	Strong Normal Phase	Methanol	Yes
			Water	Yes
Polar			Acetic acid	Yes

Analytical methods for quantitative determination of drugs in biological fluids

Numerous methods are employed for the quantitative determination of drugs in biological fluids. According to biological fluid and drug to be quantitated these methods are selected. They are:

a) Methods based on immunoassay procedure
   - ✓ Radioimmunoassay (RIA).
   - ✓ Enzyme-multiplied immunoassay technique.
   - ✓ Enzyme-linked immunosorbent assay (ELISA).

b) Microbiological methods.

c) Capillary electrophoresis.

d) Chromatographic methods.
   - ✓ Gas chromatography (GC).
   - ✓ High performance liquid chromatography (HPLC).
   - ✓ Liquid chromatography-mass spectroscopy (LC-MS).
   - ✓ Gas chromatography –mass spectroscopy (GC-MS).

The role of chromatography in bioanalytical studies:

Liquid chromatography with UV or fluorescence detector has been used successfully for drug discovery development and clinical trials. Compounds with low circulating levels or low dosed compounds such as inhaled products, a method has to be developed with necessary sensitivity could take a particular laboratory time with derivatisation schemes being required.

For shorter run times the selective ion monitoring (SIM), multiple reaction monitoring (MRM) are used to reduce need for complete resolution of analyte in chromatographic method. In order, to know the concentration of drug and its metabolites in biological fluids can be done by chromatography which are useful in preclinical, clinical and drug discovery and development process. The hyphenated techniques such as LC-MS, GC-MS are important for bioanalytical studies. In chromatography separation of particular compound is possible and quantity of the compound can be also known. The separation of compounds in chromatography is based on different principles.

ESTIMATION OF DRUGS IN BIOLOGICAL SAMPLES BY HPLC: [19-22]

High performance liquid chromatography is one of important techniques in chromatography. HPLC is an advanced form of liquid chromatography used in separating the complex mixture of molecules encountered in chemical and biological systems. HPLC is really the automation of traditional liquid chromatography under conditions which provides enhanced separations during shorter periods of time. It is ideally suited for separation and identification of amino acids, proteins, nucleic acids, hydrocarbons, carbohydrates, pharmaceuticals, pesticides, antibiotics, steroids etc. Its simplicity high specificity and wide range of sensitivity make it ideal for the analysis of many drugs in both dosage forms and biological fluids. HPLC is a versatile analytical tool useful in identification and quantitative estimation of low concentration of drugs and metabolites in biological matrices. HPLC is having many advantages most of drugs in biological matrix can be estimated by this. Some of its advantages than other methods are:

- Rapid speed.
- Improved resolution (wide variety of stationary phase).
- Precise and reproducible.
- Easy recovery of sample, handling and maintenance.

- Greater sensitivity.
- Calculations are done by integrator itself.
- Ideal for substances of low volatility.
- Reusable column

HPLC, revealed by the late Prof. Csaba Horváth for his 1970 Pittcon paper, originally presented the fact that high pressure was used to generate the flow required for liquid chromatography in packed columns. In the starting, pumps only had a pressure capability of 500 psi. This is called high pressure liquid chromatography [HPLC]. The early 1970s saw a tremendous move in technology. These new HPLC instruments could develop up to 6,000 psi [400 bars] of pressure, incorporate improved injectors, detectors and columns. High-performance liquid chromatography [HPLC] is now one of the most important tools in analytical chemistry. It has the ability to separate, identify and quantitate the compounds that are present in any sample that can be dissolved in a liquid. Today, compounds in less concentrations as low as parts per trillion [ppt] may easily be identified. Hence HPLC can be applied to any sample, such as pharmaceuticals, nutraceuticals, cosmetics, environmental matrices, forensic samples, industrial chemicals.

PRINCIPLE:

The principle of separation is based on the typical modes of separation of that of a classical chromatography i.e., Adsorption, Partition, Ion exchange and Gel permeation.

a) Adsorption chromatography

Here the separation of the components on the mixture is achieved by adsorption which is a surface phenomenon. It involves the accumulation of the liquid or gaseous solute on to the surface of the solid particle. The separation is based on the affinity characters of the solutes with solid (stationary phase). Compounds having less affinity with the stationary phase will be eluted faster while those compounds with high affinity with the stationary phase will elute later.

b) Partition chromatography

In this method the stationary phase will be liquid coated with solid support. Here the separation is achieved based on the relative distribution of solute between the two liquid phases i.e., based on their partition co efficient value. This type of chromatography may be either Normal phase or Reverse phase depending on the nature of mobile phase and stationary phase.

c) Ion –exchange chromatography

This method involves the reversible exchange like sign ions between the stationary ion exchange resin and liquid mobile phase. Separation is achieved due to the difference in strength of electrostatic interactions of the solutes with the stationary phase.

d) Gel permeation chromatography

Here the mechanical sorting of molecules are based on the size of the molecules in solution. Small molecules are able to permeate more pores and are, therefore, retained longer than large molecules and hence large molecules are eluted first.

HPLC is historically divided into two different sub classes based on the polarity of the mobile and stationary phases.

1. Normal phase high performance liquid chromatography
2. Reverse phase high performance liquid chromatography

- **Normal phase high performance liquid chromatography:**

Techniques in which the stationary phase is more polar than the mobile phase is call normal phase high performance liquid chromatography.

- ✓ Stationary Phase –Polar nature e.g.: SiO_2, Al_2O_3.
- ✓ Mobile Phase – Non-polar nature e.g.: Heptane, hexane, cyclohexane, $CHCl_3$, CH_3OH
- ✓ Mechanism:
- Polar compounds travels slower and eluted slowly due to higher affinity b/w solute and stationary phase.

- No polar compound travels faster and eluted first due to lower affinity b/w solute and stationary phase.
- This technique is not widely used in pharmaceutical separation.

➢ **Reverse phase high performance liquid chromatography:**

Techniques in which the mobile phase is more polar than the stationary phase is called reverse phase high performance liquid chromatography.

✓ Stationary phase – Non-polar nature. e.g.: n-octadecyl, n-octyl, ethyl, phenyl diol

✓ Mobile Phase – polar nature. e.g.: Methanol or Acetonitrile/water or buffers

✓ Mechanism:

- A polar compound travels faster and eluted first due to lesser affinity b/w solute and stationary phase.
- Non polar compounds travel slower and eluted slowly due to higher affinity b/w solute and stationary phase.

HPLC INSTRUMENTATION

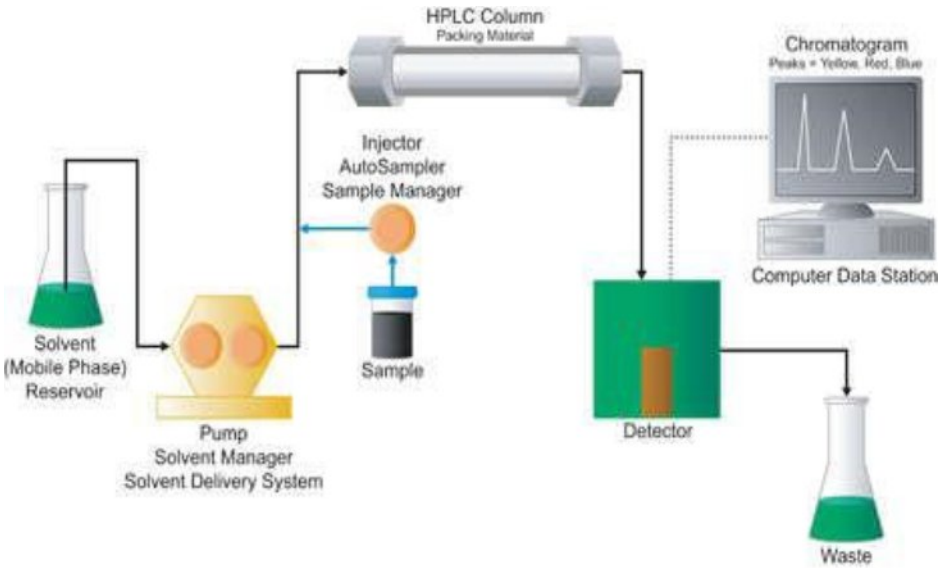


Fig 4: Schematic diagram of HPLC

The essential parts of HPLC are:

- Solvent reservoir
- Pumps
- Injector
- Column
- Detector
- Recorder

WORKING PROCEDURE

- A liquid mobile phase is pumped under pressure through a stainless steel column containing particles of stationary phase with a diameter of 3-10μm.
- Degassing and filtration is needed to remove dissolved air and to eliminate suspended particles and organic impurities
- The pumps passes mobile phase through column at high pressure and at controlled flow rate.
- The analyte is loaded onto the head of the column via a loop valve.
- Syringe is used to inject the sample through a self sealing inert septum directly into the mobile phase .Sample is introduced in the column without causing interruption to mobile phase flow.
- Guard column is placed anterior to the separating column and it serves as a protective factor that prolongs the life and usefulness of the column.
- The separation of a mixture occurs according to the relative lengths of time spent by its components in the stationary phase.
- The resolving power of a column increase with column length and the number of theoretical plate per unit length.
- Smaller the particles size of the stationary phase, the better the resolution.
- Solute molecule having more affinity towards the stationary phase elutes later and lesser affinity gets eluted faster.
- Monitoring of the columns effluent can be carried out with a variety of detectors

.

Quantitative analysis by HPLC: [23- 25]

For quantitative of analysis in HPLC generally three methods are used. They are

1) External standard method.
2) Internal standard method.
3) Standard addition method.

External standard method*:*

In this method standard and sample peak area or height are directly compared. In external standard method can be done by using single standard or up to three different standard solutions. In this method standard and unknown sample are injected and then the concentration of unknown sample can be determined by plotting calibration curve graphically or by numerically using response factors. For good quantitation in this method constant chromatographic conditions should be maintained for both standard and sample separation.

Internal standard method:

In this method a known quantity of a compound is added to known amount of sample to give separate peaks, which compensates the loss of compound of interest during sample pretreatment. In order to overcome various analytical errors addition of internal standard is commonly is used quantitation method. The compound selected for internal standard should be completely separate from the sample and should not interfere it. In chromatographic analysis internal standard is added to the compound to be analyzed are desirable, if any loss of compound may occur during handling.

In this way both internal standard and concentration of sample ratio remains constant regardless of amount of solution lost. Any loss of compound of interest will be accompanied by the loss of an equivalent fraction of internal standard. The internal standard selected should have similar properties of that of the interested compound or any other compound with other properties also can be taken. In chromatographic analysis internal standards are frequently used.

The internal standard used should be added to sample before sample preparation and mixed properly. By using response factor (R_f) concentration of sample can be known.

Necessary of internal standard

- ✓ It should elute closely to the interested compound.
- ✓ No interferences should present in a completely resolved peak.
- ✓ Stable one, unreactive with compound of interest, mobile phase and column packing.
- ✓ Behaved equally to the compound of interest for analysis like pretreatment, derivative formation.etc
- ✓ Not be present in the original ample.
- ✓ Available in high purity.
- ✓ It should be added at a concentration, which gives peak area or peak height equal or unity with the compound of interest.

Standard addition method:

In this method for the sample solution known amount of standard is added. By plotting calibration curve graphically the quantitative analysis is carried out. This method is used for sample which contains which contains very small quantity of element to be analyzed. In trace analysis also this method is used. By adding the standard to sample solution the peak area is increased and concentration of sample will be computed by interpretation.

BIOANALYTICAL METHOD VALIDATION (BMV): [26-28]

A bioanalytical method is a set of procedures involved in the collection, processing, storage, and analysis of a biological matrix for a chemical compound. Bioanalytical method validation (BMV) is the process used to establish that a quantitative analytical method is suitable for biomedical applications. Method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum, or urine, is reliable and reproducible for the intended use.

Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the method are suitable and reliable for the intended analytical applications.

Bioanalytical method validation is vital not only in terms of regulatory submission but also for ensuring generation of high quality data during drug discovery and development. BMV assures that the quantification of analyte in biological fluids is reproducible, reliable and suitable for the application. Method validation is a process that demonstrates that the method will successfully meet or exceed the minimum standards recommended in the Food and Drug Administration (FDA) Guidance for accuracy, precision, selectivity, sensitivity, reproducibility, and stability of the developed method.

Need for Bioanalytical Method Validation:

The reason for validating a bioanalytical procedure is to demonstrate the performance and reliability of a method and hence the confidence that can be placed on the results. It is mandatory that all bioanalytical methods must be validated if the results are used to support registration of a new drug or the reformulation of an existing one. Validation involves documenting, through the use of specific laboratory investigations, that the performance characteristics of the method are suitable and reliable for the intended analytical applications.

1. It is essential to used well-characterized and fully validated bioanalytical methods to yield reliable results that can be satisfactorily interpreted.

2. It is recognized that bioanalytical methods and techniques are constantly undergoing changes and improvements; they are at the cutting edge of the technology.

3. It is also important to emphasize that each bioanalytical technique has its own characteristics, which will vary from analyte to analyte, specific validation criteria may need to be developed for each analyte.

4. Moreover, the appropriateness of the technique may also be influenced by the ultimate objective of the study. When sample analysis for a given study is conducted at more than one site, it is necessary to validate the bioanalytical method(s) at each site and provide appropriate validation information for different sites to establish inter-laboratory reliability.

Types of Bioanalytical Method Validation:

A. **Full validation:**

The full validation is an establishment of all validation parameters to apply to sample analysis for the bioanalytical method for each analyte. It is important

a) When developing and implementing a bioanalytical method for the first time
b) For a new drug entity
c) When metabolites are added to an existing assay for quantification then Full validation is done.

B. **Partial validation:**

These are modifications to bioanalytical methods for which Full validation is not necessary. Modifications done for bioanalytical methods such as, small changes like change in species with matrix(from rat plasma to mouse plasma),change in matrix with in a species(from human urine to human plasma)change in laboratories or analysts, instruments, change in sampling process procedures, change in analytical method like changing detector.

C. **Cross validation:**

In this two bioanalytical methods are compared. The "reference" method which is original one is compared with the revised one "comparator". This is done where two bioanalytical methods are compared and from that same data is prepared for study. This is done in two ways. Spiked matrix samples and subjected samples validation done at sane site or done at different sites i.e. different laboratories or by using different techniques in same laboratory.

FDA guidelines on bioanalytical method validation: [29, 30]

For bioanalytical method validation FDA had given some guidelines. These guidelines are given the validation performed was to be accurate. They are:

- Analyte stability in biological matrix at intended storage and operating conditions should be kept.
- Standard curve for matrix-based one should contain minimum 5 standards without including blank and it should cover the entire range of concentrations expected.
- Essential parameters that are performed for acceptability of bioanalytical method are precision, accuracy, selectivity, sensitivity and reproducibility.
- Then the lower limit of quantitation should serve as lowest concentration on the standard curve and that is not confused with limit of detection.
- All these parameters are to be defined during the Full validation of a bioanalytical method.

Validation parameters: [31-34]

The common parameters used in the bioanalytical validation is given as follows,

1. Accuracy
2. Precision
    a. Repeatability
    b. Intermediate Precision
    c. Reproducibility
3. Linearity / Range
4. Specificity / Selectivity
5. Limit of Detection (LOD)
6. Limit of Quantification (LOQ)
7. Range
8. Robustness
9. Ruggedness.
10. System suitability

1) Accuracy:

It is the closeness of mean tests results obtained by the method to true concentration of analyte. It is also named as trueness. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. Most commonly used method for determination of accuracy is recovery studies. The usual range is being 10% above or below the expected range of claim. The % recovery was calculated using the formula,

$$\%\,\text{Recovery} \quad \frac{(a+b)-a}{bX100}$$

Where,

a – Amount of drug present in sample

b – Amount of standard added to the sample

Acceptance Criteria:

- In assay method, mean recovery will be 100% ± 2% at each concentration between the ranges of 80-120% of the target concentration.
- In impurity method, mean recovery will be 0.1% absolute of the theoretical concentration or 10% relative, whichever is greater for impurities between the ranges of 0.1-2.5 % (V/W).

2) Precision:

When the procedure is applied repeatedly to multiple samplings of single homogenous sample under prescribed conditions then precision, is a closeness of individual measurements of the analyte. It is done at three levels such as repeatability, intermediate precision, and reproducibility.

Repeatability: It expresses precision under same operating conditions i.e. with in the laboratory same analyst using same equipment over a short period of time.

Intermediate precision: It is the precision under different laboratory conditions i.e. varying only in different analyst, on different days, or using different equipments within the same laboratory.

Reproducibility: It is the precision between different laboratories and is often determined in method transfer experiments

Acceptance Criteria:

- ✓ Percentage Relative deviation (%RSD) NMT 1 % (Instrument precision)

- ✓ (%RSD) NMT -2% (Intra- assay precision)

3) Linearity:

As per ICH definition "the ability to obtain test results which are directly proportional to the concentration of an analyte within given range is known as linearity of an analytical procedure". By using correlation coefficient this can be tested. Using correlation coefficient is a benefit as it is a relationship between concentration and response data. In this data is analyzed by linear least square regression co-efficient and b of the linear equation,

$$Y = aX + b$$

By the above equation regression **r** value can be known. For the method to be linear the **r** value should be close to1. Where **Y** is the measured output signal, **X** is the concentration of sample, **a** is the slope, **b** is the intercept.

Acceptance criteria:

Coefficient of correlation should be NLT 0.99.

4) Specificity / selectivity:

A method is said to be specific when it produces proper response only for a single analyte. It can be demonstrated by performing Placebo / blank interference and forced degradation studies. If the expected impurities or related substances are available, then they should be analyzed along with the analyte or sample to check the system suitability, retention factor, tailing factor and resolution etc. In this peak purity studies are done for specificity.

5) Limit of detection (LOD):

The limit of detection is the lowest concentration of analyte in the sample which can be detected but not quantified under given experimental conditions. The lowest concentration which can be distinguished from the background noise with a certain degree of confidence is defined as limit of detection. Prepare the blank solution as per test method and inject six times into the chromatographic system. Similarly prepare the linearity solution staring from lowest possible concentration of analyte to 150 % (or as per protocol) of target concentration and establish the linearity curve.

The detection limit (DL) may be expressed as:

$$LOD = \frac{3.3 \times \text{Standard deviation of the response of the blank } (\sigma)}{\text{Slope}}$$

The slope shall be estimated from the calibration curve of the analyte.

6) Lower Limit of quantification (LLOQ):

It is also the lowest concentration of analyte in the sample but quantitatively determined with suitable accuracy and precision.

In calibration curve it is the lowest concentration point. It is determined by accuracy by the presence of background signal and by precision i.e. reproducibility of analyte in the method.

$$LOD = \frac{10 \times \text{Standard deviation of the response of the blank } (\sigma)}{\text{Slope}}$$

Acceptance Criteria:

- In Pharmaceutical application, the LOQ is typically set at minimum 0.05% for active pharmaceutical ingredients.
- LOQ defined as the lowest concentration providing a RSD of 5%.
- LOQ should be at least 10% of the minimum effective concentration for clinical applications.

7) Range:

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. The range of a bioanalytical assay is the concentration interval over which an analyte can be measured with acceptable precision and accuracy.

8) Robustness:

It is the measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

9) Ruggedness:

Ruggedness according to the USP is "the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions, such as different labs, different analysts, and different lots of reagents. The following are the typical method parameters need to test during method validation:

- Analyst-to-analyst variability.
- Column-to-column variability.
- On different days.
- In different laboratories

Table 2: Method Validation Requirements for Example (ICH)

METHOD VALIDATION REQUIREMENTS	ACCEPTANCE CRITERIA
Precision	
Assay repeatability	≤ 1% RSD
Intermediate precision (Ruggedness)	≤ 2% RSD
Accuracy	
Mean recovery per concentration	100.0% ± 2.0%
Limit of detection	
Signal to-to-noise ratio	≥ 3:1
Limit of quantification	
Signal to-to-noise ratio	≥ 10:1
Linearity/Range	
Correlation coefficient	>0.99
y-Intercept	± 10%
Visual	Linear
Robustness	
System suitability met	yes
Solution stability	± 2% change from time zero
Specificity	
Resolution from main peak	>2 min. (retention time)

System suitability:

System suitability testing is an integral part of analytical procedures. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated. The simplest form of system suitability test involves a comparison of the chromatogram trace with a standard trace. This allows a comparison of the peak shape, peak width, and baseline resolution.

These are the parameters that can be calculated experimentally to provide a quantitative system suitability test report:

- Number of theoretical plates (efficiency)
- HETP
- Capacity factor,
- Peak asymmetry factor
- Resolution,
- Tailing factor

System Suitability Parameters and Recommendations (ICH Guidelines)

Table 3: System suitability parameters and recommendations

Parameter	Formula	Recommendation
Theoretical Plates (N)	$N=5.54*(t_R/W_{0.5})^2$	In general should be > 2000
HETP(H)	H= L/N	HETP↓ Column efficiency↑
Peak asymmetry (As)	As= b/a	In general it should be 1
Tailing Factor (T)	T= (b+a)/2a	In general it should be 1
Resolution (R)	$R_s \quad \frac{2(t_2 - t_1)}{W_1 + W_2}$	R_s of > 2 between the peaks of interest.

APPLICATION OF A VALIDATED BIOANALYTICAL METHOD TO ROUTINE [35]

In general, biological samples can be analyzed with a single determination without duplicate or replicate analysis of the assay method has acceptable variability as defined by the validation data, This is ture for procedures where precision and accuracy variability's routinely fall within acceptable tolerance limits.

The following recommendations should be noted in applying a bioanalytical method to routine drug analysis:

- ✓ A matrix-based standard curve should consist of a minimum of five to eight standard points, excluding blanks (either single or replicate), covering the entire range.
- ✓ Response Function: Typically, the same curve fitting, weighting and goodness of fit determined during pre-study validation should be utilized for the standard curve within study. Response function is determined by appropriate statistical tests based on the actual standard points during each run in the validation.
- ✓ The QC samples should be used to accept or reject the run. These QC samples are matrix spiked with analyte.
- ✓ System suitability: Based on the analyte and technique, a specific standard operating procedure (or sample) can be identified to assure the optimum operation of the system employed.
- ✓ Any required sample dilutions must utilize like matrix (e.g. human to human) obviating the need to incorporate actual within-study dilution matrix QC samples.
- ✓ Repeat Analysis: It is important to establish an SOP or guideline for repeat analysis and acceptance criteria. This SOP or guideline should explain the reasons for repeating sample analysis.

Bioanalysis and the production of Pharmacokinetic, toxic kinetic and metabolic data plays a fundamental role in the pharmaceutical research and development involved in the drug discovery and development process. Therefore the data must be produced to the acceptable scientific standards and specifications lay by the different regulatory agencies worldwide. Bioanalytical methods must be validated to objectively demonstrate the fitness for their intended use.

2. LITERATURE REVIEW [36-47]

SLNO:	AUTHOR	NAME OF THE JOURNAL	TITLE OF THE ARTICLE	ANALYTICAL CONDITIONS
1	Rajendra B. Kakde *et al*.,	Journal of Chromatographic Science (2013)	Stability-Indicating **RP -HPLC** Method for the Simultaneous Determination of Escitalopram Oxalate and Clonazepam	**SP**: ODS Hypersil C18 column (250,x 4.6 mm) **MP**: Mixture of acetonitrile 50 mM & phosphate buffer 10 mM,triethylamine (70:30 v/v) **FR**: 1.0 ml/min λ: 268 nm
2	Bhimanadhuni *et al*.,	International Current Pharmaceutical Journal (2012)	Development and validation of an **RP-HPLC** method for the simultaneous determination of Escitalopram Oxalate and Clonazepam in bulk and its pharmaceutical formulations	**SP :** Hypersil ODS C18 column (250 mm X 4.6 mm; 5µ) **MP:** Mixture of buffer and acetonitrile (50:50 v/v) **FR:** 1.0 ml/min λ : 240 nm

3	R. D. Chakole *et al*.,	International Journal of Phytopharmacy (2012)	Simultaneous estimation of escitalopram and clonazepam by **RP-HPLC** in various pharmaceutical formulations	**SP** : C18 column (250 × 4.6 mm, 5 µ) **MP**: Mixture of methanol and buffer of pH 4.0 (90:10 v/v) **FR**: 1.0 ml/min λ : 248 nm
4	Bhosale Suryakant D *et al*.,	Journal of pharmaceutical and biomedical sciences (*2012*)	**RP-HPLC** Method for simultaneous estimation of Escitalopram Oxalate and Etizolam in a tablet formulation	**SP:** HiQ-sil C18 HS column (250 x 4.6 mm) **MP:** Mixture of methanol and phosphate buffer pH-5 (70:30,v/v) **FR**: 1.0 ml/min λ : 254 nm
5	Lasan V M *et al*.,	International Journal for Pharmaceutical Research Scholars (2012)	Analytical Method Development and Method Validation for Escitalopram Oxalate in Pharmaceutical Dosage Forms by **HPLC** Method	**SP :** Inertsil ODS-2 C18 column (250 x 4.6 mm, 5 µm) **MP:** Mixture of potassium dihydrogen phosphate buffer (pH 3.8 adjusted with OPA) and acetonitrile and methanol (670:280:50v/v/v) **FR:** 1.0 ml/ min λ : 238 nm

6	Tapobana samanta *et al*.,	International journal of chemistry research (2011)	**RP-HPLC** method for the estimation of escitalopram in bulk and in dosage forms	**SP :** Xterra RP C18 column (150 mm ×4.6 mm, 5 µm) **MP :** Mixture of phosphate buffer pH 7.0 and an organic mixture acetonitrile and methanol (55: 45 v/v) **FR :** 1.2ml/min **λ :** 238nm
7	Carlos E.M. de SOUSA *et al*.,	Latin American Journal of Pharmacy (2012)	Determination of Escitalopram in Human Plasma by High Performance Liquid Chromatography-Tandem Mass Spectrometry (**LC-MS**)	**SP**: ACE C18 Column (125 x 4,6 mm) **MP**: Mixture of acetonitrile and 0.2 % formic acid in water (60:50 v/v) **Detection Method**: Triple quadrupole mass spectrometer equipped with an electrospray ionization source for mass detection

8	Pallavi alegete *et al*.,	International journal of pharmacy (2016)	Simultaneous quantification of Risperidone and Escitalopram oxalate in human plasma by **LCMS/MS**: application to a pharmacokinetic Study	**SP**: x-terra RP8 column (50 mm × 4.6, 5µm) **MP**: Mixture of acetonitrile:ammonium acetate buffer 5 mm, pH 5.0±0.05 **Detection method**: Tandem mass spectrometric assay
9	Mahadeo V. Mahadik	Eurasian Journal of Analytical Chemistry (2007)	Application of Stability Indicating **HPTLC** Method for the Quantitative Determination of Escitalopram Oxalate in Pharmaceutical Dosage Form	**SP**: TLC aluminium plates precoated with silica gel 60F-254 **MP**:Mixture of Toluene: Acetone: Ethanol: Ammonia (5:1:1:0.2 v/v) **Detection method**: analysis at 239 nm

10	Bhumika D. Sakhreliya *et al*.,	Journal of Pharmaceutical Science and Bioscientific Research (JPSBR) (2012)	Development and Validation of **U V** Spectrophotometric Methods for Simultaneous Estimation of Escitalopram oxalate and Etizolam in their Combined Tablet Dosage Form	**Solvent** : 0.1 N HCl **Method I** : simultaneous equation method λmax of Escitalopram oxalate 238.2 nm and λmax of Etizolam 251.6 nm **Method II** : Q ratio (absorbance ratio) method 238.2 nm and 248.8 nm (isoabsorptive point) **Method III**: absorbance correction method absorbance measurement at 238.2 nm for Escitalopram oxalate and 292.8 nm for Etizolam
11	Santhosh D.shelke *et al*.,	Journal of Chemical and Pharmaceutical sciences (2009)	Development and validation of **U V** Spectrophotmetric method of escitalopram oxalate in bulk and pharmaceutical formulations	**Solvent**: 0.1 N HCl **λmax** :239 nm
12	Sharma S *et al*.,	Journal of Young Pharmacists (2010)	Zero order – **UV** spectrophotomteric method for estimation of escitalopram oxalate in tablet formulations	**Solvent** : 80% v/v aqueous methanol **λmax** : 238 nm

3. DRUG PROFILE [48-53]

ESCITALOPRAM OXALATE

1. Chemical profile:

Synonyms:

Escitalopram, (s) - citalopram, (+) - citalopram

Brand name:

Lexapro, Estilo, Citalex, Nexito, Etilopram

Chemical name/ IUPAC Name:

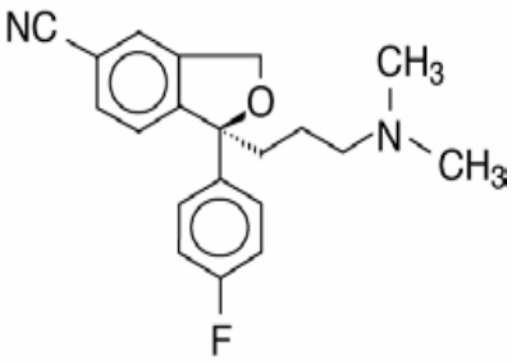
S-(+)-1-[3-(dimethyl-amino) propyl]-1-(p-fluorophenyl)-5-phthalancarbonitrile oxalate.

Molecular formula:

$C_{20}H_{21}FN_{2O}{\cdot}C_2H_2O_4$

Molecular mass: 414.40 g/mol

Structure:



$.C_2H_2O_4$

Fig 5: Molecular structure of escitalopram oxalate

Pharmacokinetic data:

Bioavailability : 80% relative to an intravenous dose

Metabolism : Hepatic (N-de methylation reaction using CYP3A4 and CYP2C19 enzymes)

Protein binding : ~56%

Half life : 27-32 hours

Excretion : Renal (10%)

Routes : Oral

Description:

Escitalopram oxalate occurs as a fine, white to slightly-yellow powder and is freely soluble in methanol and dimethyl sulfoxide (DMSO), soluble in isotonic saline solution, sparingly soluble in water and ethanol, slightly soluble in ethyl acetate, and insoluble in heptane .

Category:

Selective serotonin reuptake inhibitor (SSRI) which is under the category of antidepressant drugs.

Dose:

For the treatment of depression it is basically 10-20 mg/ day and for elderly persons it will be 5-10 mg/day. Escitalopram tablets (LEXAPRO) are film-coated, round tablets contains escitalopram oxalate in strengths equivalent to 5 mg, 10 mg, and 20 mg escitalopram base.

Indications:

Labeled indications include major depressive disorder (MDD) and generalized anxiety disorder (GAD). Unlabeled indications include treatment of mild dementia-associated agitation in nonpsychotic patients

2. Pharmacological profile:

Mechanism of action:

The antidepressant, antiobsessive-compulsive, and antibulimic actions of escitalopram are presumed to be linked to its inhibition of CNS neuronal uptake of serotonin. Escitalopram blocks the reuptake of serotonin at the serotonin reuptake pump of the neuronal membrane, enhancing the actions of serotonin on $5HT_{1A}$ auto receptors. Antidepressant action of escitalopram is linked to potentiation of serotonergic activity in the central nervous system (CNS) resulting from its inhibition of CNS neuronal reuptake of serotonin (5-HT). Escitalopram also does not bind to, or has low affinity for, various ion channels including Na+, K+, Cl-, and Ca++ channels .Similar to other SSRIs, Escitalopram also bind with significantly less affinity to histamine, acetylcholine, and norepinephrine receptors than tricyclic antidepressant drugs.

Pharmacodynamics:

Escitalopram is one of a class of antidepressants known as selective serotonin reuptake inhibitors (SSRIs). It is used to treat the depression associated with mood disorders. It is also used on occassion in the treatment of body dysmorphic disorder and anxiety. The antidepressant, antiobsessive-compulsive, and antibulimic actions of escitalopram are presumed to be linked to its inhibition of CNS neuronal uptake of serotonin. In vitro studies show that escitalopram is a potent and selective inhibitor of neuronal serotonin reuptake and has only very weak effects on norepinephrine and dopamine neuronal reuptake. Escitalopram has no significant affinity for adrenergic (alpha1, alpha2, beta), cholinergic, GABA, dopaminergic, histaminergic, serotonergic ($5HT_{1A}$, $5HT_{1B}$, $5HT_2$), or benzodiazepine receptors; antagonism of such receptors has been hypothesized to be associated with various anticholinergic, sedative, and

cardiovascular effects for other psychotropic drugs. The chronic administration of escitalopram was found to downregulate brain norepinephrine receptors, as has been observed with other drugs effective in the treatment of major depressive disorder. Escitalopram does not inhibit monoamine oxidase**.**

Drug interactions:

Some medicines may interact with escitalopram like, Buspirone, Fenfluramine derivatives, anticoagulants, some diuretics, Tramadol, Carbamazepine or Cyproheptadine etc as some has risk of seizures may be increased or some may decrease Escitalopram's effectiveness.

Adverse reactions:

Signs of overdose include convulsions, coma, dizziness, hypotension, insomnia, nausea, vomiting, sinus tachycardia, somnolence and ECG changes (including QT prolongation).

4. AIM AND OBJECTIVE

Escitalopram is one of the novel antidepressant drugs belonging to the group of selective serotonin reuptake inhibitors (SSRI) for the treatment of various affective disorders.

The drug dosage is individualized by maintaining the plasma or blood drug concentration within the targeted therapeutic window. It is important to understand the relationship between plasma and tissue concentrations of drugs and the factors that can cause variations in drug concentration. Therapeutic Drug Monitoring provides an indirect measurement of drug concentration at effector sites in tissue compartments of interest. Hence, there is a need to develop simple, rapid, reliable and cost effective analytical method for the clinical routine drug monitoring and pharmacokinetic study.

HPLC being a versatile analytical tool for the identification and quantitative estimation of low concentration of drugs and metabolites in biological matrices, the present study aims to develop

- A precise, accurate, rapid and sensitive analytical method for the estimation of Escitalopram oxalate in human plasma.

- A simple and precise, solid phase extraction for sample preparation.

5. PLAN OF WORK

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD:

Bioanalytical method development for Escitalopram oxalate:

The present work is planned to two phases.

- **PHASE I**
    1. **Optimization of chromatographic conditions**
        - Selection of wavelength
        - Selection of initial separation conditions
        - Selection of mobile phase (pH, peak modifier, solvent strength, ratio and flow rate)
        - Selection of the stationary phase
        - Selection of internal standard
        - Sample preparation by solid phase extraction method
        - Estimation of escitalopram oxalate in human plasma
- **PHASE II**

    2. **Validation of the developed method**

        The developed method were proposed to be validated using various validation parameters such as,

        - Accuracy
        - Precision
        - Linearity
        - Limit of Detection (LOD)
        - Lower Limit of Quantitation (LLOQ)
        - Selectivity / Specificity
        - System suitability.
        - Ruggedness

6. METHODOLOGY

- **MATERIALS AND INSTRUMENTS USED:**

a) Drug sample and study products:

Escitalopram oxalate and Tinidazole were procured from Aurobindo Pharma, Hyderabad, India

Plasma: Blank plasma was collected from Hi-tech laboratory, SITRA, Coimbatore

b) Chemicals and solvents used :

- ✓ Acetonitrile (HPLC grade)
- ✓ Methanol (HPLC grade)
- ✓ Ortho phosphoric acid (Analytical grade)
- ✓ HPLC grade water was prepared by using Millipore MilliQ water purification system.

c) Instruments used:

- ✓ Elico pH meter LI 127.
- ✓ Shimadzu LC-20 AT HPLC.
- ✓ SPD-M20A Prominence diode array detector.
- ✓ Shimadzu 1600 LC-UV Spectrophotometer.
- ✓ Sonica ultrasonic cleaner.
- ✓ Solvent filtration unit – Millipore.
- ✓ Shimadzu electronic balance AY 220.
- ✓ Ultra cooling centrifuge – Remi, India
- ✓ SPE catridge – strata ™-X (Phenomenex)

- **METHOD DEVELOPMENT AND OPTIMIZATION OF CHROMATOGRAPHIC CONDITIONS FOR THE ESTIMATION OF ESCITALOPRAM OXALATE**

i. Selection of Wavelength:

An UV spectrum of 10 µg/ml escitalopram oxalate in methanol was recorded by scanning in the range of 200nm to 400 nm. From the UV spectrum, wavelength of **240nm** was selected as the drug showed good absorbance at this wavelength.

ii. Selection of chromatographic method :

Selection of proper chromatographic method depends on the nature of sample properties like ionic/ ioinizable /neutral character, its molecular weight and solubility. The drug selected for the study was polar in nature. Hence Reverse phase HPLC or ion-pair or ion exchange chromatography must be used. The RP-HPLC method was selected for the initial separations because of its simplicity and suitability.

iii. Initial chromatographic conditions for separation of Escitalopram oxalate:

Thousand µg/ml of escitalopram oxalate was prepared by dissolving 10 mg of escitalopram oxalate in 10 ml of HPLC grade methanol. A gradient run was performed for the initial separation. From this the appropriate ratio of the organic phase and the aqueous required to elute the analyte from the column was determined. An aliquot of the mixed standard solution containing 10 µg/ml of drug was prepared and chromatographed using the following chromatographic conditions:

- Stationary phase : C8/C18 column
- Mobile phase : Solvent A – 0.2% Ortho Phosphoric Acid
  Solvent B – Acetonitrile
- Solvent ratio : Gradient run, 10 to 100 % solvent B
- Flow rate : 0.8 to 1.5 ml/min
- Sample injector : Rheodyne 7125 injector
- Sample size : 20 µl
- Temperature : Room temperature (25ºC)

From the above gradient run, the approximate percentage of acetonitrile in the 0.2% ortho phosphoric acid solution required to elute the drugs from the column was determined. This ratio was used for subsequent isocratic separation and the chromatogram was recorded.

To optimize the chromatographic conditions, the effect of chromatographic variables such as mobile phase pH, solvent strength, flow rate, solvent ratio and the nature of stationary phase on the peak separation were studied. The resulting chromatograms were recorded and the chromatographic parameters such as capacity factor, asymmetric factor, and resolution and column efficiency were calculated. The conditions that give the best resolution, symmetry and capacity factor were selected for the estimation.

a) Effect of pH

The standard solution was chromatographed for 20 minutes using acetonitrile or methanol in buffer solutions of different pH ranging from 2.0-7.0 (ortho phosphoric acid solutions and phosphate buffer of pH 2.0 and 3.0) as the mobile phase at a flow rate of 1.5 or 1 ml/min.

b) Effect of nature of stationary phase

Different reversed phase stationary phases (C8 and C18) were used and the chromatograms were recorded. When C8 columns were used, the retention time and the separation between the peaks were reduced. Hence C18 column is preferred.

c) Effect of solvent strength

Different mobile phases, namely, acetonitrile and methanol were used at a flow rate of 1.5 or 1 ml/min. The ratio of acetonitrile or methanol was substituted by the other solvents; their solvent to buffer ratios was calculated. The resulting ratios of the mobile phase were prepared and the drugs were chromatographed.

d) Effect of ratio of the mobile phase

The standard solution was chromatographed with mobile phases of different ratios of organic and aqueous phases at a flow rate of 1.2 and 1 ml/min.

e) Flow rate

The standard solution was chromatographed at different flow rates namely 1.2, 1.0 and 0.9 ml/min.

f) Selection of internal standard

Based upon polarity and solubility, tinidazole, ornidazole, and diclofenac sodium were selected and chromatographed along with the standard drug. The elution time of tinidazole was 4.7 min. The peak of tinidazole was symmetric and well resolved from the peak of the escitalopram. Hence, for the present study tinidazole was selected as the internal standard.

PRETREATMENT METHOD FOR BIOLOGICAL FLUID:

Method of sample preparation is an important criterion for biological samples. For the present study plasma was obtained from HI- TECH Clinical Laboratory Services, Coimbatore. Solid Phase Extraction (SPE) method was selected for the present study.

Preparation of Standard solutions:

Ten milligrams of standard escitalopram oxalate and internal standard were separately dissolved in the few ml of methanol and made up the volume with methanol. Serial dilutions were made with mobile phase to get a final concentration of 1 mg/ml.

Preparation of working standards:

From the stock solution 1 ml was taken and diluted to 10 ml(100 µg/ml), from this solution 1 ml was further diluted to 10 ml(10µg/ml).From this 1ml was taken and diluted to 10 ml(1µg/ml) from the resulting solution 1ml was taken and diluted to 10 ml gives100 ng/ml solution.100, 200, 400 ,600, 800 ng/ml solutions were prepared by

diluting 1.0 ml, 2.0 ml, 4 ml, 6 ml and 8 ml respectively. In the similar way the working standards were prepared for internal standard also.

Plasma spiking procedure:

Hundred micro litre of drug and internal standard were added to 0.5 ml plasma and vortexed for 2 mins. To this 1 ml ACN was added and again vortexed for 2-3 mins. It was then centrifuged at 3000 rpm for 5 mins and the supernatant was collected and refrigerated until analysis.

Extraction by SPE

Solid Phase Extraction (SPE) is the most powerful sample preparation technique in common use now a day, where it involves the use of chromatographic sorbent in a column format.

Before sample extraction the solid phase extraction cartridge were conditioned with 1.0ml of methanol followed by 1.00ml of purified water and 1.00ml of 2% ortho phosphoric acid buffer solution. Prepared plasma samples were then loaded on to the SPE cartridges and eluted completely under slow vacuum and the cartridges were then washed with 1.00 ml 2% ortho phosphoric acid buffer solution and 1.00 ml of methanol and allowed to dry. The analyte and the internal standard were then eluted with 1.00ml acetonitrile. The eluted samples were then injected into the HPLC system.

The chromatograms of plasma extracted with methanol, acetonitrile and methanol–acetonitrile mixture, were recorded using the fixed chromatographic conditions. The chromatogram of blank plasma without any drug was also recorded. Based up on the percentage recovery acetonitrile was selected as eluting solvent for the present study because of its higher percentage recovery.

Preparation of Standard Graph:

Preparation of calibration standards:

SPE cartridge was conditioned with 1.0 ml of methanol followed by 1.0 ml of purified water and 1.0 ml of 2% ortho phosphoric acid buffer solution. Prepared plasma samples were then loaded on to the SPE cartridges with the drug concentration of 100,200,400, 600 and 800 ng/ml respectively. The cartridges were then washed with 1.00 ml 2% ortho phosphoric acid buffer solution and 1.00 ml of methanol and allowed to dry. The drug was then eluted with1.0ml acetonitrile. A quantity of 20 µl was injected into the HPLC column and chromatograms were recorded. Standard calibration graph was plotted using ratio of peak area of escitalopram oxalate to its concentration.

ESTIMATION OF ESCITALOPRAM OXALATE IN HUMAN PLASMA:

Recording the chromatogram:

The optimized chromatographic conditions were maintained to record the chromatograms of the calibration standards of escitalopram oxalate and internal standard. First, baseline stabilization was done for about 20 minutes. Then standard solutions, calibration standard solutions and sample from clinical study containing escitalopram oxalate were injected and chromatograms were recorded.

VALIDATION OF THE METHOD

After developing a method its validation is necessary to prove the suitability of the method for the intended purpose. Here the procedure followed for the validation of the developed method is described.

a) **Precision*:***

Intraday and interday precision studies were conducted. In intraday precision plasma sample containing drug at three different concentrations with internal standard

were injected and chromatogram was recorded. Similarly interday precision over a two week period time was evaluated.

Acceptance criteria:

RSD of the mean concentration of five readings should be less than 15% for bioanalytical method.

b) Accuracy:

It is the closeness of mean tests results obtained by the method to true concentration of analyte. It is also named as trueness. In this studies the selected concentration of the plasma were injected six times and mean peak area for each concentration was calculated. Concentration of the each injection was calculated and the standard deviation between the readings is calculated. To bioanalytical study the percentage RSD should be less than 15%.

c) Recovery studies:

The relative recovery of drug from plasma was calculated by comparing the readings of concentration obtained from the drug spiked plasma to that of equal concentration from standard sample. Recovery studies were carried out six times for sample concentration at three levels within the calibration curve.

Acceptance Criteria:

For an assay method, mean recovery should be 85-105% ± 2%.

d) Linearity and Range:

Linearity and range were estimated by using calibration curve. By using calibration standards prepared by spiking plasma (Escitalopram oxalate) and internal standard (Tinidazole) at different concentrations like 100ng/ml to 800ng/ml the calibration graph was plotted taking concentration of spiked plasma on x-axis and

peak area on y-axis. The linearity is determined from 50% to 150% of the proposed concentration.

Acceptance Criteria:

Coefficient of correlation of the calibration should be not less than 0.99

e) Lower Limit of Quantification (LLOQ):

The LLOQ is determined by using the calibration curve. Limit of quantitation is the concentration of substance in the sample that will give a signal-to-noise ratio of 10:1. Detection limit corresponds to the concentration that will give a signal-to-noise ratio of 3:1. The signal to noise ratio were performed by comparing measured signal of blank plasma sample with those of known low concentration of drug.

f) Specificity:

Specificity of the method was demonstrated by using diode array detector peak purity test. The diode array spectrum of both standard and sample peak were recorded and compared. The other way for doing specificity based in measurement of absorbance ratio of drug peaks at two different levels. The retention time (Rt), resolution factor (Rs) and tailing factor (T) were noted for the peaks of escitalopram oxalate. Peak purity study is done to prove that a developed method is specific for the drug of interest.

Acceptance criteria:

Purity angle should be less than purity threshold i.e.0.99-1.00

g) Stability:

Stability of the sample, standard and reagent used in HPLC method is required for a reasonable time to generate reproducible and reliable results. Stability of plasma sample spiked with drug were subjected to three freeze-thaw cycles, short term stability at room temperature for 3 hours and long term stability at -20^0C for four weeks. In addition, stability of standard solution and internal standard were performed at room

temperature for 6 hours and under frozen condition for two weeks. The stability of this solution was studied by performing the experiment and looking for changes in separation, retention and asymmetry of the peak which were then compared with the pattern of chromatogram of freshly prepared solutions.

h) Selectivity:

Selectivity is the analytical method ability to differentiate and quantify the analyte in the presence of other components in the sample. The selectivity was established by two different methods.

Method I: Chromatograms of six blank plasma samples were compared with chromatogram obtained from standard solutions. Each chromatogram was tested for interferences due to endogenous plasma component on the retention times of the selected drugs.

Method II: This method involves the peak purity test method using diode array detector. The PDA spectrum, UV spectrum, absorbance ratio curve and first derivative spectrum of the standard and sample peaks was recorded using PDA detector and compared for the peak purity of drug.

i) Robustness:

The robustness of the method was studied by changing the chromatographic conditions slightly. The standard solutions were injected in these changed chromatographic conditions.

- ± 1 % difference in the ratio of acetonitrile in the mobile phase.
- ± 0.5 difference in units of pH of the buffer.
- ± 1 % difference in flow rate of the mobile phase.

In these changed conditions the separation factor, retention time and peak symmetry was calculated. Deviation in results from original run should be less than 2%.

j) System suitability studies:

In system suitability studies certain parameters were calculated namely, column efficiency, resolution, capacity factor by repeated injection of standard solutions. As specified in the USP these systems suitability studies were carried out.

Capacity factor (k') it is measurement of sample molecule how good is retained by a column during separation. The ideal k value ranges from 2-10.

$$\text{Capacity Factor (k')} = V_1 - V_0 / V_0$$

Where, V_1 is the retention volume at the apex of the peak (solute) and V_0 is the void volume of the system.

Resolution (Rs) is the difference between the retention times of two solutes divided by their average peak width. The ideal value of (Rs) is 1.5

$$\text{Resolution (Rs)} = Rt_1 - Rt_2 / 0.5(W_1 - W_2)$$

Where, Rt_1 and Rt_2 are the retention times of component 1 and 2, respectively.

Column Efficiency (N) of a column is measured by the number of theoretical plates per meter. For ideal good separation, column efficiency N value ranging from 5,000 to 100,000 plates/meters.

$$\text{Column efficiency (N)} = R_t^2 / W^2$$

Where R_t is the retention time and W is the peak width.

Peak asymmetry factor- For better column performance it was calculated by the formula. When asymmetry factor of value 0.9 to 1.1 then it is achievable for a well packed column.

$$\text{Peak asymmetry factor (As)} = b / a$$

Where a and b are the distances on either side of the peak midpoint.

Table 4: System suitability parameters and recommendations

Parameter	Recommendation
Capacity Factor (k')	The peak should be well-resolved from other peaks and the void volume, generally k' > 2.0
Repeatability	RSD < / = 1 % for N > / = 5 is desirable.
Relative retention	Not essential as long as the resolution is stated.
Resolution (R_s)	R_s of > 2 between the peak of interest and the closest eluting potential interferent (impurity, excipient, degradation product, internal standard, etc.)
Tailing Factor (T)	T of < / = 2
Theoretical Plates(N)	In general should be > 2000

7. RESULTS AND DISCUSSION

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

A bioanalytical RP-HPLC method was developed for the escitalopram oxalate. The chromatographic conditions were stabilized in order to provide a good performance of the assay. The standard and internal standard solutions were prepared and chromatograms were recorded. The study proposes a method for the determination of escitalopram oxalate by solid phase extraction using RP-HPLC.

Optimization of chromatographic conditions:

i) Selection of wavelength:

The sensitivity of a HPLC method depends upon the proper selection of the wavelength. The overlay spectrum of escitalopram oxalate is given in Figure **(Fig 6)**

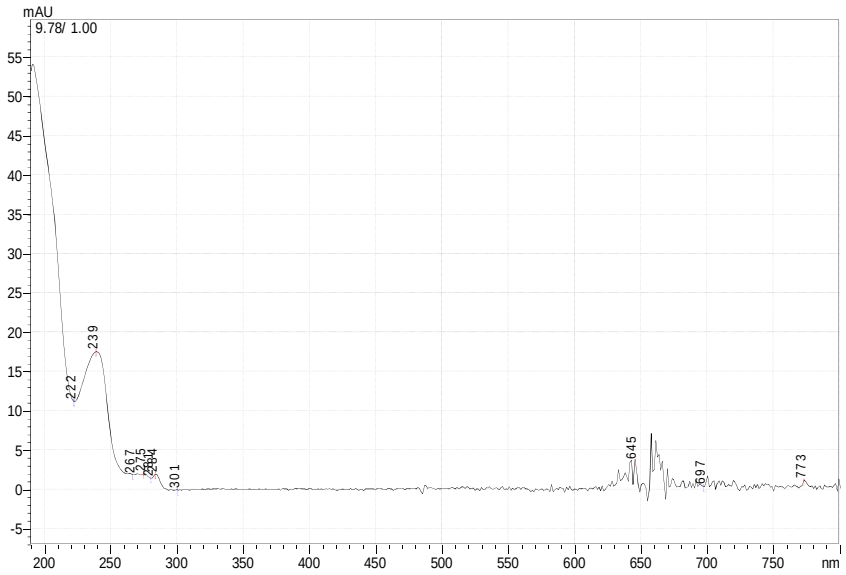


Fig 6: Overlay spectrum of Escitalopram oxalate

From the UV spectrum, wavelength of **240 nm** was selected. At this wavelength the drug showed good absorbance.

ii) Initial chromatographic conditions:

Acetonitrile was selected as organic phase to elute the drug from the stationary phase because of its favorable properties. For the HPLC separation 0.2% ortho phosphoric acid solution was selected as aqueous phase because of the nature of the selected analytes. The ratio of organic phase and mobile phase was altered to get better separation and resolution of the compounds.

Phosphate buffer of pH 2 was tried as aqueous phase for the better result, but there is no much difference in the peak shape and intensity. So acidic solution of 0.2% ortho phosphoric acid was fixed as aqueous phase and the ratio was altered step by step to get a proper resolution and sharp peak.

- **Chromatographic condition – 1**

Stationary phase	:	Enable C18 column
Mobile phase	:	Solvent A – water
		Solvent B - Acetonitrile
Solvent ratio	:	50: 50 (A: B)
Detection Wavelength	:	240 nm
Flow rate	:	1.0 ml/min
Sample size	:	20 µl

For the above chromatographic condition no significant peak was obtained hence an acid mobile phase was selected for next trial.

- **Chromatographic condition – 2**

Stationary phase	:	Enable C18 column
Mobile phase	:	Solvent A – 0.2% OPA (O-Phosphoric acid)
		Solvent B - Acetonitrile
Solvent ratio	:	50: 50 (A: B)
Detection Wavelength	:	240 nm
Flow rate	:	1.0 ml/min
Sample size	:	20 µl

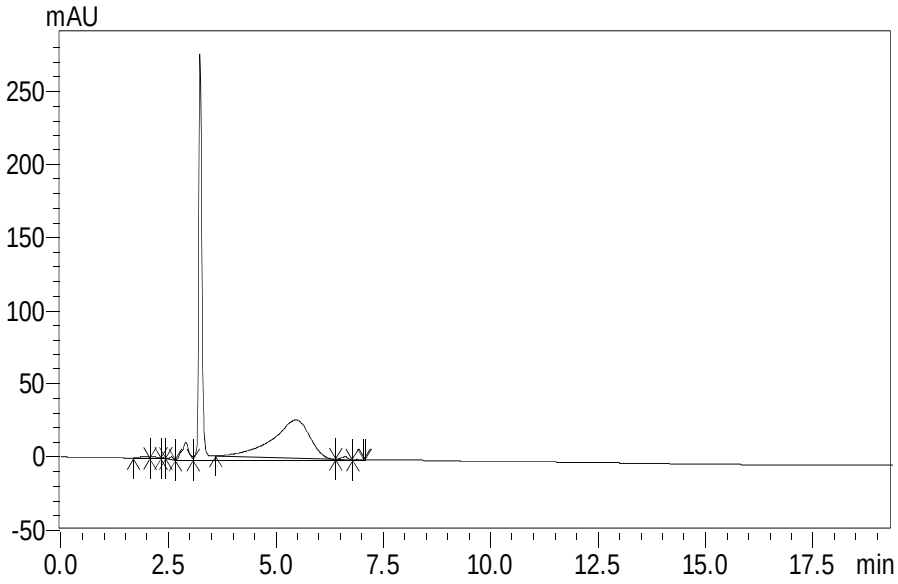


Fig 7: Chromatogram of escitalopram oxalate

At the above chromatographic conditions escitalopram oxalate was eluted at the retention time of 2.5 min. The peak observed posses asymmetric peak characteristics, hence not selected for the study

- **Chromatographic condition – 3**

Stationary phase	:	Enable C18 column
Mobile phase	:	Solvent A – 0.2% OPA(O-Phosphoric acid)
		Solvent B - Acetonitrile
Solvent ratio	:	60: 40 (A: B)
Detection Wavelength	:	240 nm
Flow rate	:	1.0 ml/min
Sample size	:	20 µl

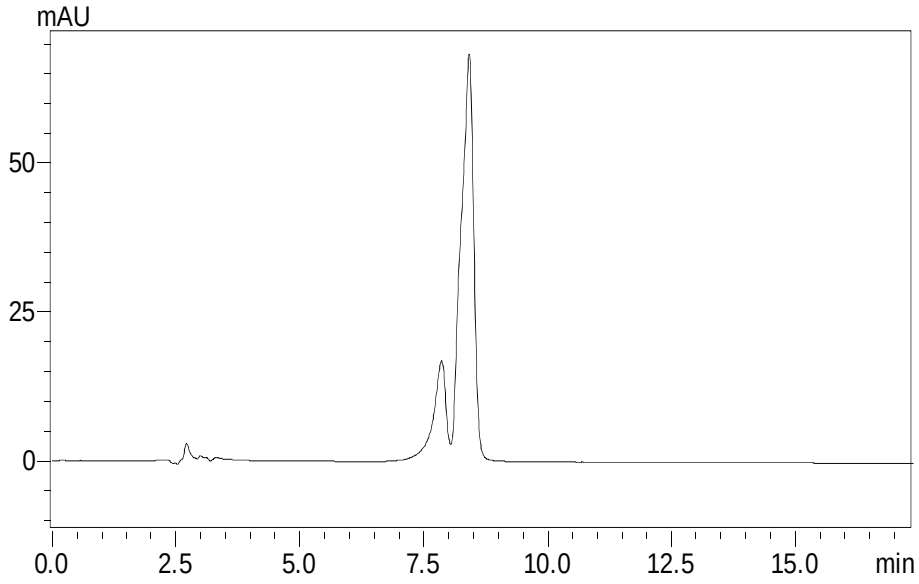


Fig 8: Chromatogram of escitalopram oxalate

At the above chromatographic conditions escitalopram oxalate was eluted at the retention time of 7.8 min. For the further improvement of chromatographic pattern i.e, to increase the retention time of escitalopram oxalate and to optimize the internal standard, the acetonitrile concentration was further decreased.

- **Chromatographic condition – 4**

Stationary phase	:	Enable C18 column
Mobile phase	:	Solvent A – 0.2% OPA (O-Phosphoric acid)
		Solvent B - Acetonitrile
Solvent ratio	:	65: 35 (A: B)
Detection Wavelength	:	240 nm
Flow rate	:	1.0 ml/min

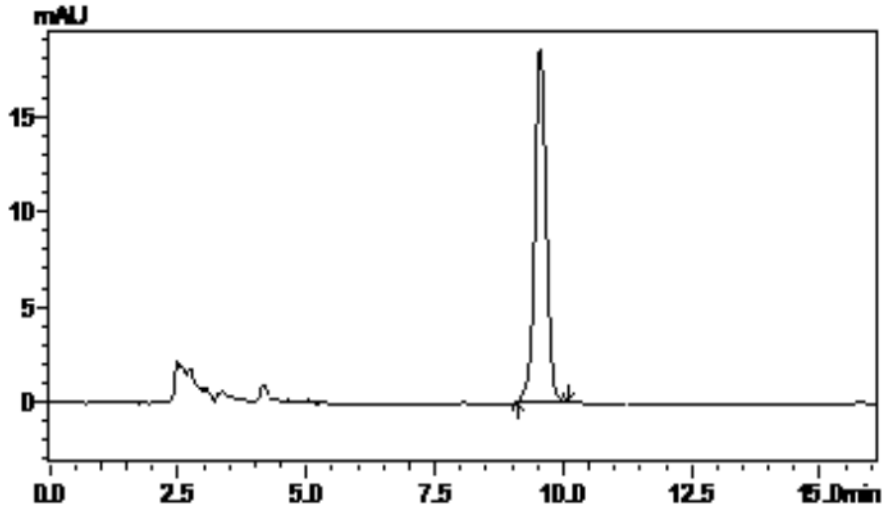


Fig 9: Chromatogram of escitalopram oxalate

Escitalopram was eluted at 10.5minutes. Peaks were also well resolved and symmetric with perfect peak properties, hence selected for further studies

iii) Selection of internal standard

Based upon polarity and solubility, tinidazole, ornidazole, and diclofenac were selected and chromatographed along with the standard drug. The elution time of tinidazole was 4.9 min. The peak of tinidazole was symmetric and well resolved from the peak of the escitalopram. Hence, for the present study tinidazole was selected as the internal standard.

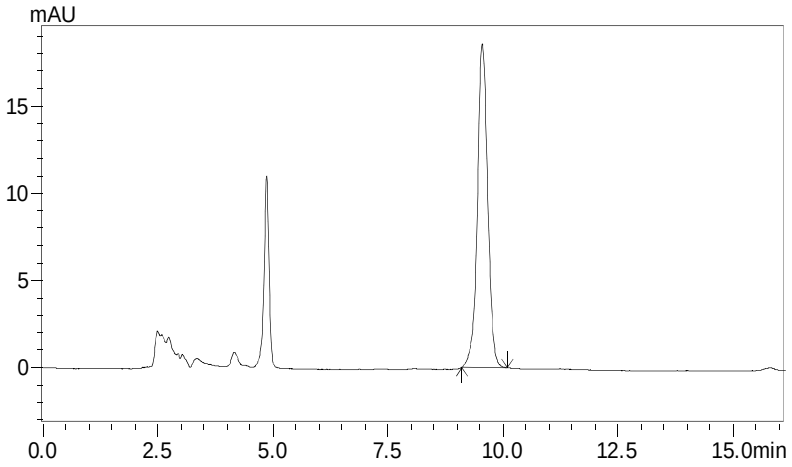


Fig 10: Chromatogram of both internal standard and escitalopram

FIXED CHROMATOGRAPHIC CONDITION

Stationary phase	:	Enable C_{18} column
Mobile phase	:	Solvent A – 0.2% Ortho Phosphoric Acid in water
		Solvent B – Acetonitrile
Solvent ratio	:	65:35 (A: B)
Detection Wavelength	:	225 nm
Flow rate	:	1.2 ml/min
Sample size	:	20 µl
Needle wash	:	HPLC grade water followed by methanol

Temperature : Room temperature (25ºC)

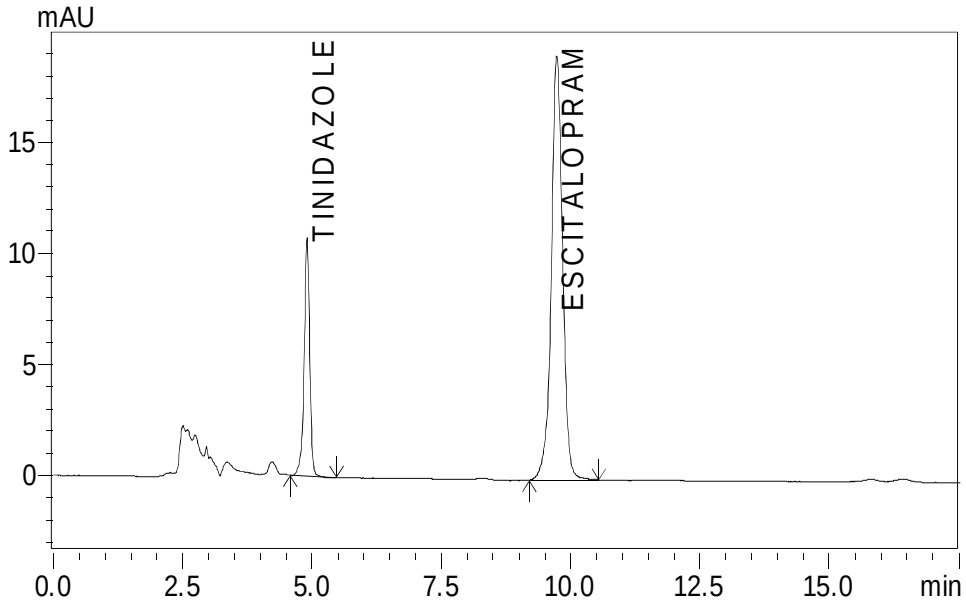


Fig 11: Typical chromatogram obtained for the tinidazole and escitalopram oxalate

The retention time of escitalopram oxalate and internal standard (tinidazole) was 4.9 and 10.5 min respectively with percent RSD of less than 2%. The results are shown in table **(Table 5)**. The peak purity study reveals that signal ratios (relative absorbance at different wavelengths) were constant across the peak profile of escitalopram oxalate. The peaks obtained in the present study were symmetric, good and no interference was observed between the peaks. The method developed was advantageous than the reported methods by its lesser precision values and increased accuracy values.

Table 5: Retention time of escitalopram oxalate and tinidazole (IS)

S. No	Method	Retention time of Drugs (min)	
		Internal Standard (Tinidazole)	**Escitalopram oxalate**
1	RP-HPLC	4.988	10.560
2		4.982	10.563
3		4.987	10.559
4		4.985	10.551
5		4.990	10.568

Chromatographic separation of Escitalopram oxalate in biological fluid:

The chromatogram of the blank plasma was recorded at the fixed chromatographic conditions and shown in figure (**Fig 12)**

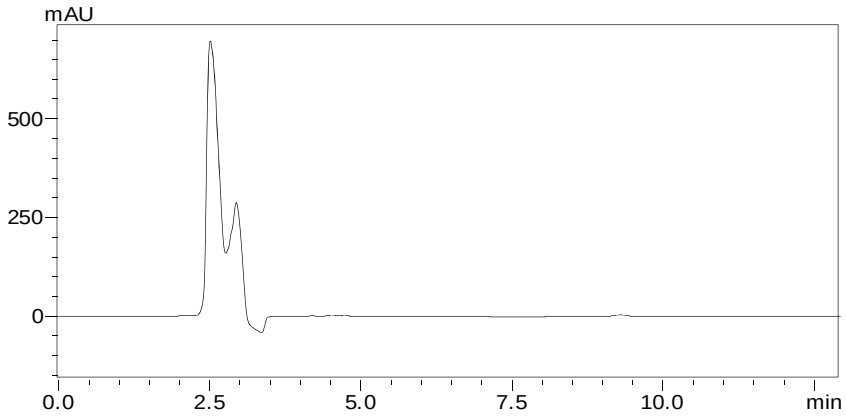


Fig 12: Chromatogram of blank plasma

Various eluting solvents were used for extraction of Escitalopram oxalate in human plasma. The eluting solvents used were methanol, acetonitrile and mixture of methanol and acetonitrile, maximum recovery percentage was found in acetonitrile. The percentage recovery was calculated and shown in **Table 6.**

The extraction method used for the present study was simple and newer than previous methods. For present study ACN is used as the eluting solvent because of its maximum recovery of drug from plasma and it is advantageous.

Table 6: Recovery study of escitalopram oxalate

Level	Conc. of drug added (ng/ml)	Amt. of drug recovered from plasma (ng/ml)			% Recovery		
		Methanol	ACN and methanol mixture	ACN	Methanol	ACN and methanol mixture	ACN
I	50	32.4	34.4	48.2	64.8	68.8	96.4
II	100	74.6	78.2	97.2	74.6	78.2	97.2
III	150	121.4	118.5	147.4	80.9	78.9	98.2

C:\Documents and Settings\Administrator\Desktop\23-3-16\TINI ESM STD 006.lcd

Acquired by	: Admin
Sample Name	: COMBAINED 23-3-16
Sample ID	: TINI AND ESM STD
Vail #	:
Injection Volume	: 20 ul
Data File Name	: TINI ESM STD 006.lcd
Method File Name	: 28-3-16.lcm
Batch File Name	:
Report File Name	: Default.lcr
Data Acquired	: 3/28/2016 12:43:17 PM
Data Processed	: 3/28/2016 1:00:56 PM

<Chromatogram>

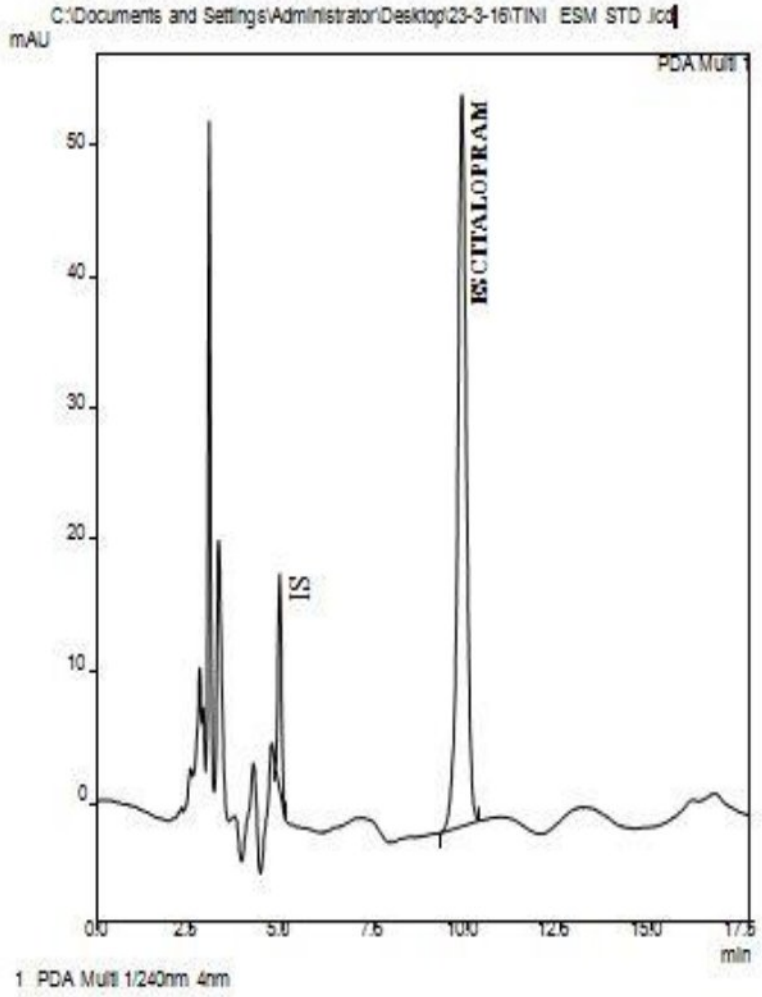


Fig 13: Chromatogram of escitalopram oxalate and internal standard without SPE

METHOD VALIDATION

a) Accuracy and precision:

At two –levels these accuracy and precision studies were conducted i.e. intra-day and inter-day. In this the present developed method, shown the good accuracy and precision. Accuracy ranges from 99.2% to100.3 % with the precision 3.57% to 5.34% in intra-day method. In inter-day method the accuracy ranges from 99.5% to 100.08% with the precision 5.78% to 6.31%. Finally the data obtained here, was found to be within limits as per ICH guidelines and method was accurate.

Intra-day studies: In this plasma concentration 100-800 ng/ml were injected six times and mean peak area was calculated separately for each concentration and from that accuracy and precision percentage RSD values were calculated and shown in table **(Table 7)**

Table 7: Accuracy and precision studies of escitalopram oxalate (Intraday)

Sl.no	Conc. of drug (ng/ml)	Mean peak Area*	Accuracy (%)	RSD (%)
1.	200	199526	99.2	4.98
2	400	312919	100.3	3.57
3	600	429303	99.9	5.32

*Average of six determinations.

Inter-day studies: In this the plasma concentrations of 100-800 ng/ml were injected into HPLC six times in three different days and mean peak areas were calculated and from that accuracy and precision percentage RSD were calculated and shown in table **(Table 8).** The percentage relative standard deviation of precision for Escitalopram oxalate was less than 15% for the bioanalytical study. The results obtained were within limits.

Acceptance criteria: *The percentage RSD value should be less than 15% for bioanalytical study.*

Table 8: Accuracy and precision studies of escitalopram oxalate (Interday)

Sl.no	Conc. of drug (ng/ml)	Mean peak Area*	Accuracy (%)	RSD (%)
1	200	191206	99.6	5.78
2	400	336188	99.2	6.31
3	600	419568	100.08	6.11

*Average of three determinations.

b) Linearity and range:

This method proved to be linear between ng/ml of Escitalopram oxalate in human plasma, with a typical calibration curve of correlation equation y = 0.06x + 0.216, correlation coefficient > 0.999 shown in table **(Table 9)**

Table 9: Calibration standards peak area

Concentration (ng/ml)	Peak area of drug	Peak area of IS	Response factor
0	0	106856	0
100	123953		1.160
200	196849		1.847
400	308814		2.913
600	434903		4.078
800	546034		5.221

The chromatograms of the plasma calibration standards with concentrations 100,200,400,600 and 800ng/ml were recorded and shown in figures **(Fig 14)** and their peak areas of both drug and internal standard were noted. The calibration curve for Escitalopram oxalate was plotted as peak response Vs concentration of the Escitalopram oxalate calibration standards in plasma was shown **(Fig : 15-19).**

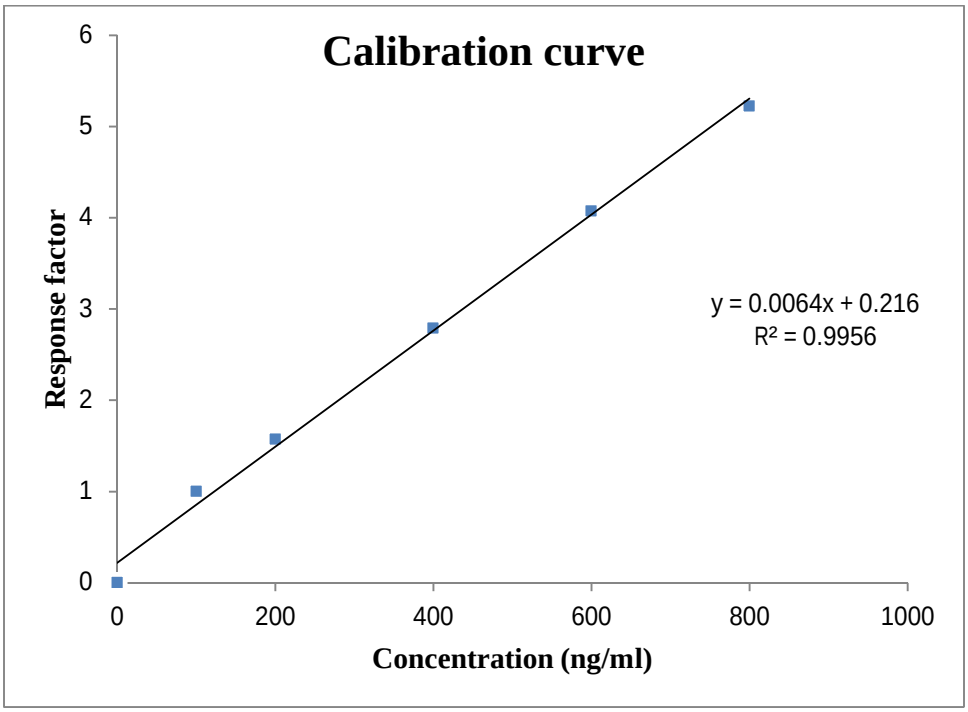


Fig 14 - Calibration curve for escitalopram oxalate

As we were using internal standard peak response was calculated for calibration curve. Peak response is the ratio of internal standard peak area to drug peak area. The correlation coefficient of escitalopram oxalate shown was 0.995 which was within limits. This calibration curve plotted was linear and showed that the method had adequate sensitivity to the concentration (100-800ng/ml) of the drug. Finally the data obtained, in this was within limits. Coefficient of correlation of Escitalopram oxalate was found to be less than 0.99.

Acceptance criteria: *The correlation coefficient should not less than 0.99*

==== Shimadzu LCsolution Analysis Report ====

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Sample ID	: TINI AND ESM STD
Vail #	:
Injection Volume	: 20 uL
Data File Name	: TINI ESM STD -1.lcd
Method File Name	: 28-3-16.lcm
Batch File Name	:
Report File Name	: Default.lcr
Data Acquired	: 3/28/2016 11:09:02 AM
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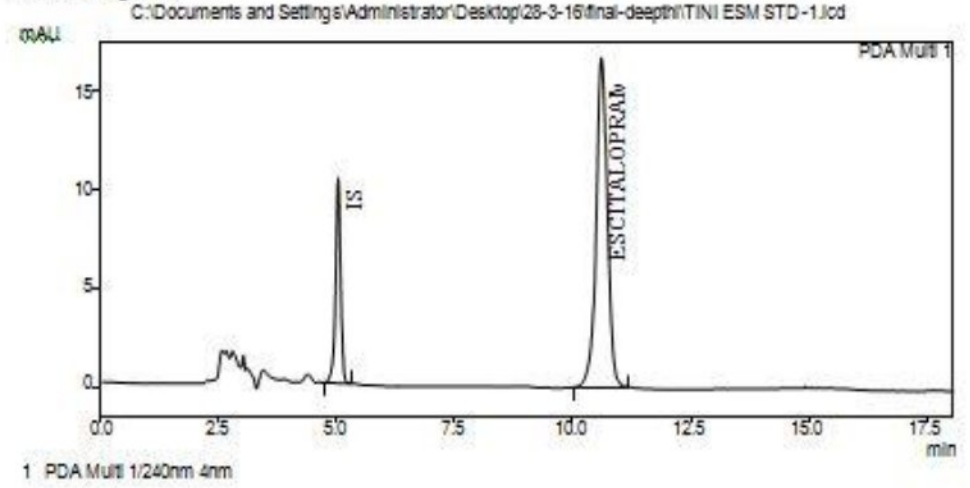

1 PDA Multi 1/240nm 4nm

PDA Ch1 240nm 4nm

PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.988	103971	10465	22.150	38.368
2	10.563	123953	16811	77.850	61.632
Total		227924	27276	100.000	100.000

Fig 15- Chromatogram of IS and Escitalopram oxalate in human plasma100ng/ml

==== Shimadzu LCsolution Analysis Report ====

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Sample ID	: TINI AND ESM STD
Vail #	:
Injection Volume	: 20 uL
Data File Name	: TINI ESM STD -2.lcd
Method File Name	: 28-3-16.lcm
Batch File Name	:
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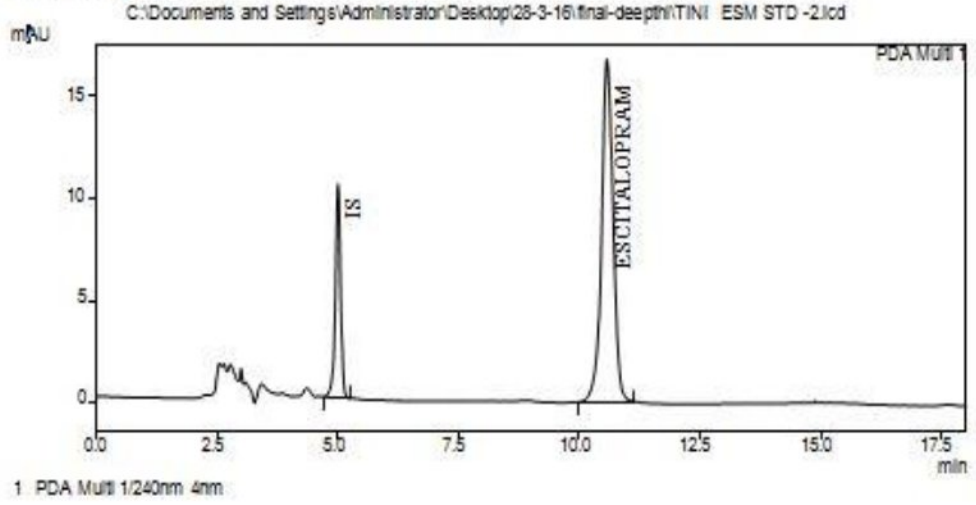

1 PDA Multi 1/240nm 4nm

PDA Ch1 240nm 4nm

PeakTable

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.988	109920	10465	22.150	38.368
2	10.563	196849	16811	77.850	61.632
Total		306769	27276	100.000	100.000

Fig 16- Chromatogram of IS and Escitalopram oxalate in human plasma 200ng/ml

==== Shimadzu LCsolution Analysis Report ====

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Vail #	:
Injection Volume	: 20 uL
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Method File Name	: 28-3-16.lcm
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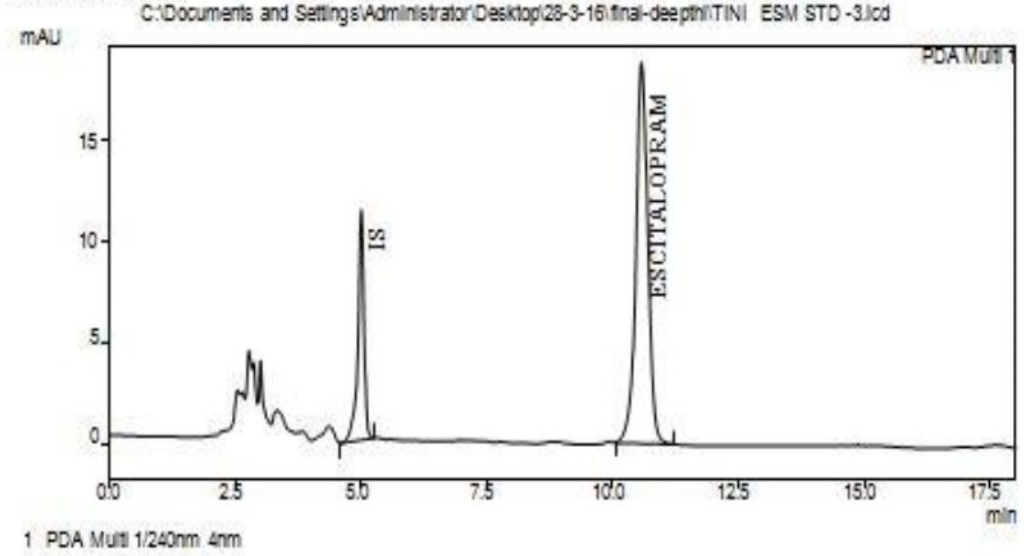

1 PDA Multi 1/240nm 4nm

PeakTable

PDA Ch1 240nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.987	107162	11434	23.572	37.666
2	10.559	308814	18922	76.428	62.334
Tota		402186	30356	100.000	100.000

Fig 17- Chromatogram of IS and Escitalopram oxalate in human plasma400ng/ml

==== Shimadzu LCsolution Analysis Report ====

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Injection Volume	: 20 uL
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Method File Name	: 28-3-16.lcm
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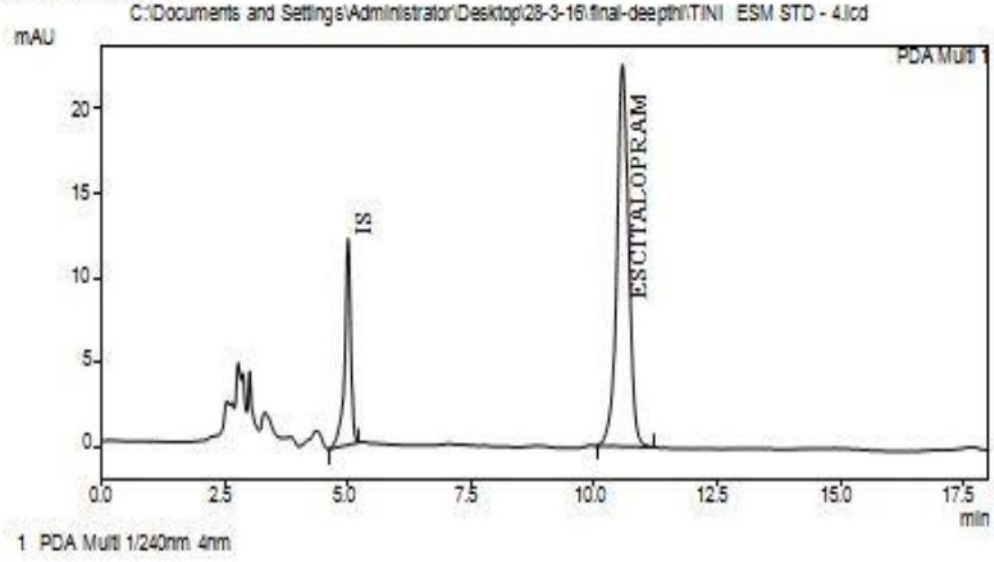

1 PDA Multi 1/240nm 4nm

PeakTable

PDA Ch1 240nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.987	105255	12226	21.850	35.111
2	10.559	434903	22594	78.150	64.889
Total		540158	34820	100.000	100.000

Fig 18- Chromatogram of IS and Escitalopram oxalate in human plasma 600ng/ml

==== Shimadzu LCsolution Analysis Report ====

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Sample ID	: TINI AND ESM STD
Vail #	:
Injection Volume	: 20 uL
Data File Name	: TINI ESM STD - 5.lcd
Method File Name	: 28-3-16.lcm
Batch File Name	:
Report File Name	: Default.lcr
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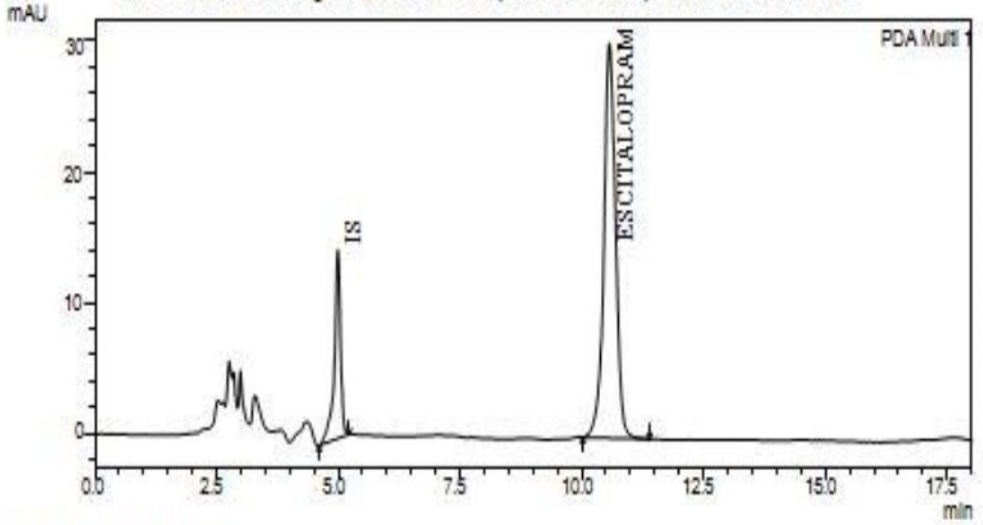

1 PDA Multi 1/240nm 4nm

PeakTable

PDA Ch1 240nm 4nm

Peak#	Ret. Time	Area	Height	Area %	Height %
1	4.987	107973	14404	20.231	32.367
2	10.559	546034	30098	79.769	67.633
Total		654007	44502	100.000	100.000

Fig 19- Chromatogram of IS and Escitalopram oxalate in human plasma 800ng/ml

c) Lower Limit of Quantification and Limit of Detection:

The LOD is the smallest concentration of the analyte which shows a measurable response. The LLOQ is the smallest concentration of the analyte, which shows response that can be accurately quantified and LLOQ = 10 × D/S and LOD = 3.3× D/S where, D is the standard deviation of y – intercepts of regression line and S is the slope of the calibration curve. This signal to noise ratio were performed by comparing measured signal of known low concentration of drug with those of blank plasma sample.

The Lower limit of quantification (LLOQ) and the Limit of Detection (LOD) for escitalopram oxalate were separately determined and reported, based on the calibration curve for spiked plasma solutions was found to be 23.79ng/ml and 7.3ng/ml respectively.

d) Recovery from plasma:

A recovery study for escitalopram oxalate in plasma using acetonitrile was shown in table **(Table 10)**. With concentrations 200ng/ml, 400ng/ml, 600ng/ml of escitalopram oxalate recovery was calculated and showed 100.2%, 99.4%, 100.4% relative recoveries and percentage RSD as 8.6%, 7.9% and 7.3% respectively.

Acceptance criteria: *For an assay method, mean recovery should be 85-105% ± 2%.*

Table 10: Recovery studies of escitalopram oxalate

Levels	Conc. of drug added (ng/ml)	Amt of drug recovered in plasma sample (ng /ml)	Percentage recovery (%)	% RSD
I	200	200.08	100.04	4.2
II	400	401.5	100.3	6.8
III	600	598.9	99.75	5.9

**Average of six determinations.

e) Ruggedness:

It expresses the precision within laboratories variations like different days, different analyst, and different equipments. Ruggedness of the method was assessed by spiking the plasma standard 6 times in two different days with different analyst and the standard solutions were analyzed by a different chemist and same instruments on a different day had been performed the reports were shown in table **(Table 11)**.

The deviation among the results obtained by two chemists on a different day was well within the limits. Hence the method was rugged.

Acceptance criteria: *The percentage RSD should be less than 15%.*

Table 11: Ruggedness studies for escitalopram oxalate

Drug	Concentration (ng/ml)	Mean peak area	%RSD
	Day I analyst – I		
Escitalopram oxalate	200	195392	2.76
	Day II analyst – II		
Escitalopram oxalate	200	199527	4.98

**Average of six determinations.

f) Specificity:

For specificity the peak purity studies were done. Here for escitalopram oxalate the peak purity index was 000 and the peak properties like peak profile were good for both standard and the sample. The peak purity and peak profiles for escitalopram oxalate standard and sample were shown in figures **(Fig 20-23)** respectively. By the data obtained in this, the present method developed was specific as values were within limits.

Acceptance criteria: *Purity angle should be less than purity threshold i.e.0.99-1.00*

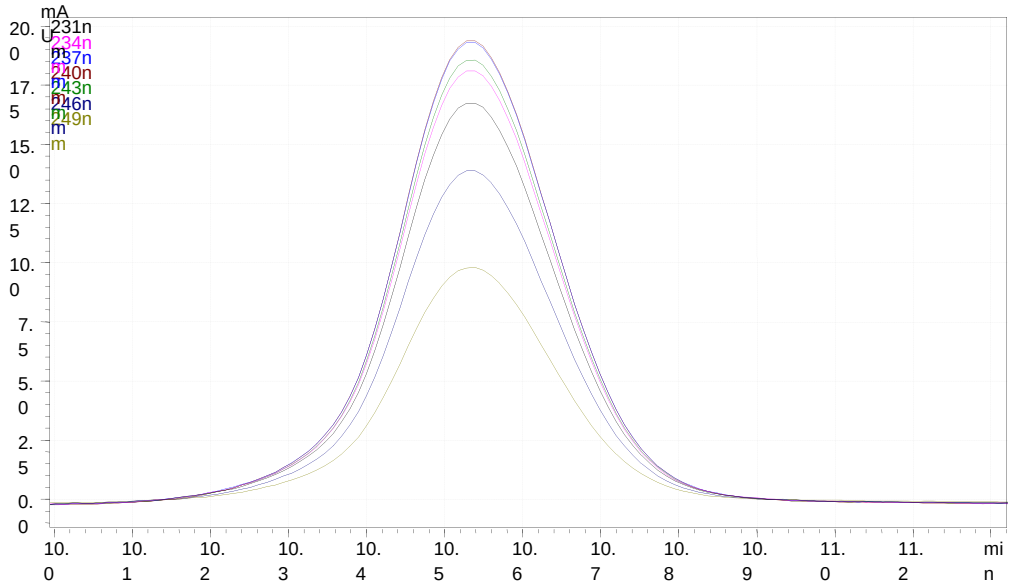

Fig 20: Peak profile of standard escitalopram oxalate

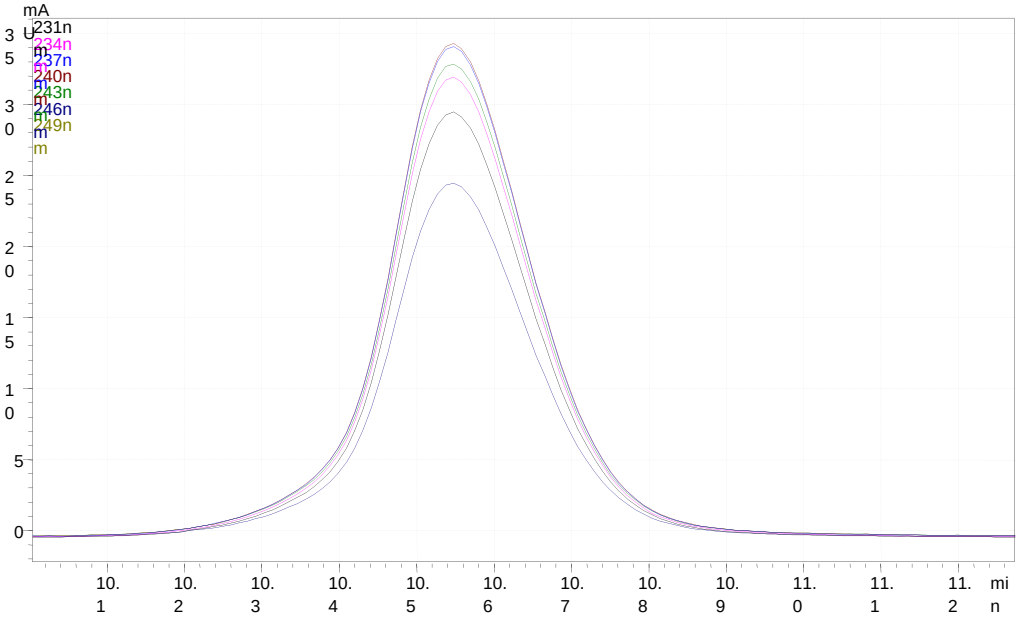

Fig 21: Peak profile of escitalopram oxalate in human plasma

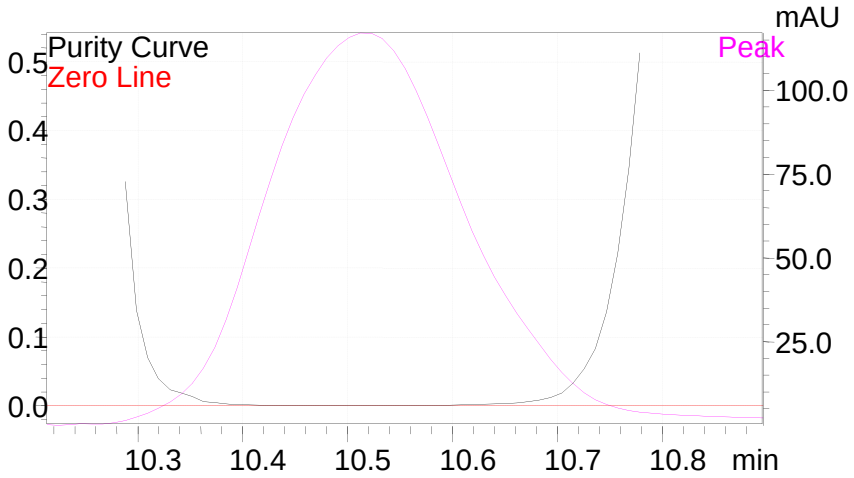

Fig 22: Purity profile of escitalopram oxalate

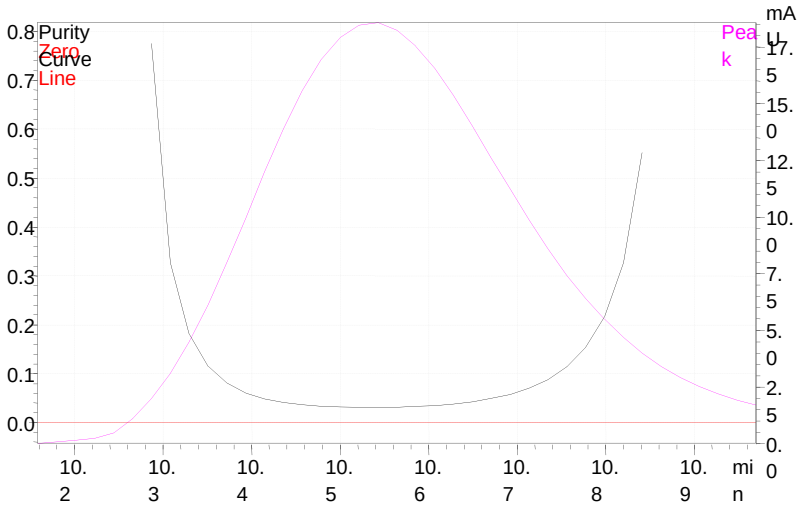

Fig 23: Purity profile of escitalopram oxalate in human plasma

g) System suitability:

These parameters were shown to be within specified limits. Column efficiency (theoretical plates), resolution factor and peak asymmetry factor, HETP, tailing factor, LLOQ are the system suitability parameters. These parameters of the optimized methods were found satisfactory. The results of the system suitability studies in plasma were shown in table **(Table 12).** These parameters were shown to be within specified limits.

Table 12: System Suitability Studies

Sl.no	Parameters	Escitalopram oxalate
1.	Theoretical Plate	9533
2.	Tailing Factor	0.995
3.	HETP	15.77
4.	LLOQ	23.79 ng/ml
5.	LOD	7.3 ng/ml
6.	Resolution	7.21
7.	K	3.17

8. SUMMARY AND CONCLUSION

A bio-analytical method was developed for the estimation of Escitalopram oxalate in human plasma by RP -HPLC method and was validated. Escitalopram is an antidepressant drug from the class called Selective Serotonin Reuptake Inhibitors (SSRI's).The method was developed using 0.2% ortho phosphoric acid and acetonitrile in the ratio of 65:35% v/v. The peaks obtained for the drug of interest and internal standard by the present method were well resolved from each other without any interference and from the plasma endogenous proteins by Solid phase Extraction method (SPE method) and the peaks were symmetrical in nature with acceptable tailing factor.

All the analytical validation parameters were determined and found in the limit as per ICH guidelines, which indicates the validity of the method .The method was validated with respect to specificity, linearity, accuracy, precision, ruggedness and robustness. The results of linearity, intraday and interday precision study and capability of the extraction method were within the limits of bioanalytical method development. The method was linear with a correlation coefficient of acceptable agreement, which is suitable for the estimation of Escitalopram oxalate in human plasma and other biological fluids.

The SPE method demonstrated relative recoveries with acceptable relative standard deviation. The lower limit of quantification (LLOQ) and limit of detection (LOD) for Escitalopram oxalate was found to be in nanograms. Hence the developed method is sensitive for the estimation of Escitalopram oxalate in trace amounts. The Peak purity studies showed that the peak purity index values were closer to unity hence reveals that the method developed was specific for the estimation of Escitalopram oxalate in blood and other biological fluids.

From the current work it was finally concluded that the developed RP-HPLC method in human plasma using solid phase extraction for sample preparation by was found to be very simple, reliable, precise, accurate, sensitive and selective analytical method for the estimation of Escitalopram oxalate . The method is suitable for routine quantitative analysis in pharmaceutical dosage forms. The method developed can be used in therapeutic drug monitoring units, bioequivalence and bioavailability studies, pharmacokinetic and toxicology studies of Escitalopram oxalate.

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